BIOLOGICAL STUDIES OF THE TUBERCLE BACILLUS

I. Instability of the Organism—Microbic Dissociation

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Plates 18 to 26

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It has become increasingly evident during recent years, that bacteria along with other living forms are subject to modification, embraced under the term of "mutation." The recognition of types of "mutation" is now general among systematic bacteriologists, as is the employment of such terms as "smooth" and "rough" to define differences in colony formation appearing in the "mutating" bacterial species. The literature on this subject has been assembled in papers and monographs and is now readily accessible so that it will not be reviewed in this paper. We refer those seeking the literature to the article by Hadley (1), but it is desirable to direct attention especially to the pioneer work in this field of Lehmann and Neumann (2), Neisser (3), Massini (4), Baerthlein (5), Arkwright (6) and DeKruif (7).

Just as the observations on bacteria in general have shown bacteria to undergo "mutation," so have the studies on tubercle bacilli, in tissue and cultures, shown them at times to be pleomorphic in structure. It is not uncommon to see in preparations "coccoid" granules, rods, beaded and branching rods. Reviews of the literature on this subject may be found in the articles on the tubercle bacillus by Kolle, Schlossberger and Pfannenstiel (8), Kahn (9) and others.

Tubercle Bacillus Cultures

Our purpose in this paper is to deal with the gross cultural characteristics of the tubercle bacillus, with special reference to the topography of colony formation and the virulence according to colony type. No mention will be made of the pleomorphism which may or may not play a part in the dissociation phenomena.

When a pathogenic acid-fast organism is cultivated on favorable media, generally the growth appears white at first. As time goes on, it becomes a cream color at the center of the crowded area and dark orange color as it becomes older. The growth may be wrinkled and

heaped in masses; or show large folds having a smooth surface; or it may be spreading and smooth. Not all cultures develop with the same rapidity; some develop profusely in a few weeks, while for others a much longer time passes before growth becomes visible.

Variations in virulence have been noted by many workers. For the last twenty years we have used the well known Saranac Laboratory human tubercle bacillus culture H_{37} for studies in infection and immunity. Up to a few years ago the virulence of this organism was very constant and apparently a small number of organisms when inoculated into guinea pigs, was sufficient to produce progressive disease. Within the last three or four years a diminution of virulence has been observed such that in some cases we failed completely to infect animals even with a heavy suspension. From time to time we have noticed a variation in antigenic properties of this organism, with special reference to the complement-fixation reaction. Some antigens were anti-complementary and low in fixing properties, while antigens prepared from a different lot of H_{37} cultures were very potent.

When the tubercle bacillus H_{37} is cultivated on liquid media, *i.e.*, veal, synthetic or potato broth, two types of growth may be seen; veil-like filament and small wrinkled heaps. The veil-like growth is confluent, spreads in very thin layers and in time covers the surface of the medium, gradually pushing its way up the side-walls of the flask. The growth coheres and it is very difficult to remove a small portion without disturbing the whole mass. Chromogenicity is generally absent. The second growth appears in small isolated islands connected by a veil-like growth. After some time it becomes very wrinkled and dry. At first it is white, and as it ages, creamy, and finally it assumes an orange color. The production of pigment is much greater in the center of the mass than at the periphery. The growth is fragile and brittle and any portion can be lifted without disturbing the adjoining one. Cultures of the first sort can be triturated to an evenly diffused suspension with no difficulty, while those of the second sort are much more difficult to put in a fine suspension.

If we start with a colony having distinct cultural characteristics, there will probably be a number of variants from the original mother colony upon subsequent cultivation. Two, perhaps three, or more variants may develop from the original colony and these may differ

from each other not only in cultural structure, but also in tinctorial characteristics, virulence and other characteristics.

In the following pages we shall describe in detail the methods used in dissociating various acid-fast organisms. Observations are recorded just as we have seen the various colonies. No attempt will be made to interpret the various stages of their development. For the earlier observations the reader is referred to previous studies by the authors (10).

Culture Media for Dissociating the Tubercle Bacillus

Gentian-violet-glycerol-egg medium with a slight modification was used throughout this investigation. The gentian-violet dye exerts some bacteriostatic action on the tubercle bacillus, a loss which is more than compensated for by the usefulness of the dye. The dye has two functions: (a) its dark color makes a satisfactory background, on which white colonies stand out prominently for observation and photography, and (b) it to some extent prevents external contamination. Even with all precautions contamination, especially by the molds, cannot be entirely prevented.

The Modified Medium.—Meat juice: 500 gm. of chopped lean veal or beef are infused in 500 cc. of 15 per cent solution of glycerine in water in a refrigerator. After 24 hours the meat is squeezed through cheese cloth and the juice is collected in a sterile beaker. *Eggs:* The shells are washed well and dried, and left in 70 per cent alcohol for ½ hour. They are removed from the alcohol and placed on a sterile towel to dry. They are then broken, emptied into a sterile beaker and mixed well with a sterile glass rod. One part of the meat juice is added to two parts of the eggs by volume. *Gentian-violet:* A 1 per cent alcoholic gentian-violet solution is added to make a final dilution 1 to 30,000 (1 cc. of dye to 300 cc. of the medium). The mixture is well stirred and filtered through sterile gauze to remove coarser particles. 35 cc. of the medium are put in Petri dishes (100 x 15 mm.), and inspissated the first day at 85°C. until completely coagulated, removed from the inspissator and put in the incubator for a gradual cooling (face down). On the second day the dishes are again put in the inspissator at 85°C. for a second inspissation for 1½ hours, after which they are again put in the incubator at 37.5°C. for 3 days to test their sterility. While still in the incubator, rubber bands are slipped around the edge of the plates and they are then removed to room temperature. This step in the preparation of the medium is very important for the following reason: At 37.5°C. the air in the Petri dishes expands and in part escapes with result that when the dish is removed to room temperature room air enters it carrying contaminating bacteria. This difficulty can be eliminated by the use of rubber bands. All plates must be incubated face down to prevent water condensation on the upper lid of the Petri dishes. We cannot emphasize too strongly the importance of this step.

Preparation of the Rubber Bands (*See Plate 18A*).—Automobile inner tubes (30 x 3½ inches), preferably new, are cut into bands 1¼ inches wide. These can be shaped into proper form by using wood discs 15 cm. in diameter and 1.5 cm. thick. The bands are stretched over the disc so that one-third will cover the sides of the disc and the remainder will cover the bottom. The discs must have a central hole of ⅜ inch. Twenty-five to thirty such discs over which the rubber bands have been stretched can be threaded on a rod through the hole, a nut put on each end and tightened, autoclaved for 1 hour at 15 pounds pressure, and cooled. The bands when removed from the discs are found to have shaped themselves so that they fit satisfactorily over the Petri dishes.

Preparation of the Suspension for Plating (*See Plate 18B*).—A small portion of a recent 12 to 14 day growth, preferably from fluid medium, is removed aseptically with a sterile platinum wire spade and triturated with the same on the inside wall of a sterile Wassermann test tube containing 3 to 4 cc. of sterile saline of pH 7.8. The trituration is done just above the fluid level. From time to time the platinum wire is dipped in the saline to carry more moisture for trituration. After complete trituration the tube is inclined and the triturated material washed into the saline. The suspension is filtered twice through two layers of sterile Whatman No. 5 filter paper. The filtrate is then diluted five to ten times for the human tubercle bacillus, while the bovine and avian require ten to twenty times. The filtrate is inoculated on the surface of five or ten plates made as described above, usually four to six drops from a capillary pipette being used for inoculation. The rubber bands are fitted around the edges of the Petri dishes. Adhesive tape may be used for labels. Rubber bands prevent desiccation of the medium as well as contamination. When a plate is taken out of the incubator, the rubber band should never be removed until it has completely cooled.

Funnels for filtration (see Plate 18B) may be made from discarded test tubes, 18 to 20 mm. in diameter. The upper portion of the funnel can be made 1½ to 2 inches long, pulled out in the flame to about 3 to 4 mm. Two layers of Whatman paper No. 5 are inserted in the funnels, suitably folded to make a cup form. For consecutive studies a number of these funnels containing the filter paper can be prepared, placed in a jar, sterilized and used as wanted.

Growth may not appear until the third week. The best time to study the colonies is after 6 to 8 weeks when they have partially matured. For the study of the finer structures of the colonies we have used a Bausch and Lomb low power binocular microscope with drum type objectives. After a plate has been removed from the incubator for study the lid of the plate may become foggy and interfere with vision. This can be remedied by warming the lid of the plate for a few seconds over a Bunsen burner.

Dissociation of the Bovine Tubercle Bacillus B_1

The bovine culture which we have successfully dissociated is known as B_1. It was isolated by Dr. E. R. Baldwin in 1904. The organism has been cultivated on

glycerine potato, glycerine egg and veal broth. The virulence has been constant for rabbits and guinea pigs until a few years ago since when it has occasionally failed to produce progressive disease in rabbits.

A suspension of 2 weeks' growth was prepared by the method described above and inoculated on the surface of gentian-violet-egg plates. After 4 weeks, isolated colonies appeared on the plates. One of the colonies (a) was perfectly round, raised, opaque, with a smooth surface resembling a moth-ball. It was easily removed from the surface. The second colony (b) was large, spreading, raised at the center, dry and wrinkled. The (a) colony was readily emulsified, while the (b) colony was suspended with difficulty (10).

The separate types of isolated colonies were emulsified, the suspensions filtered and new plates prepared. After due time, colony (a) instead of being like a moth-ball, was flat, spreading and appeared very much like a star. In the middle of each colony there was a small nipple-like growth with a smooth surface, which was surrounded by a darker pigment forming a circle and from this central zone the growth radiated in the form of large folds toward the periphery in a rosette form. The periphery of the colony was very irregular and raised. The colony (b) had a different topography, the central zone being raised with numerous irregular folds and at places appearing like a honeycomb. The periphery was flat and veil-like with perfectly smooth edges spreading over the medium. The colony (a) of the second subculture was difficult to emulsify, while (b) could be prepared in suspension very readily. The different physical properties relating to emulsification probably are due to the lipin content or they may have a different isoelectric point.

The two principal colonies which we have just described were difficult to separate at times and their characteristics were sometimes very confusing. Very often colonies appeared on the plates differing from the two colonies already described. It must be kept in mind that there may be some intermediate colonies which develop from time to time and play some part in the life cycle of the organism.

From the preliminary observations reported here we were convinced that dissociation of the tubercle bacillus does take place and that our methods up to this time were inadequate to make a further study until we could find a medium which would stabilize the development of the organism and retard the dissociation. In other words it was required for further studies that the colonies should be of uniform topography and readily separated.

Recently we have modified the medium with the object of obtaining uniform colony structure. The base of the medium was gentian-violet-egg to which sodium glycocholate and sodium taurocholate were added. Sodium taurocholate in the proportion of 0.25 per cent was found to be most suitable.

When the two colonies dissociated from the bovine tubercle bacillus

were cultivated on this medium, they presented characteristics which could be differentiated without any difficulty. One of the colonies was perfectly round, moist, like a moth-ball, opaque, with smooth surface, easily emulsified and on subsequent subcultures on plates developing a round and flat colony. The other colony was large, flat opaque, changing with age to cream yellow, moist and with smooth surface. It emulsified with difficulty and on subsequent subculturing developed flat colonies (see Plate 19, 1, 2, 34).

The former we shall now call "S" and the latter "R." Organisms of the "S" colony when inoculated into guinea pigs, regardless of the route used, produced generalized tuberculosis in a short time, the disease spreading rapidly throughout the viscera. A few drops of a suspension of 5,000,000 organisms of this colony when introduced into the conjunctiva, without injury, produced conjunctivitis and vitreous opacity with photophobia within 4 weeks. The cervical lymph nodes could be palpated and in many instances the inguinal lymph nodes could be palpated after a short time. The animals reacted to tuberculin within 12 to 15 days and the intensity of the reaction was approximately 15 x 15 mm. with a central necrotic area. Animals inoculated intracutaneously, intraperitoneally, intracardially and intratesticularly developed generalized tuberculosis and death in 3 to 6 weeks. This colony also produced rapid, progressive tuberculosis in rabbits after inoculation with a small number of organisms. The disease was very extensive and tubercle bacilli could be found in the bone-marrow 2 months after the inoculation.

When a suspension of the "R" colony was used in a strength similar to that of the "S" colony, it was found that the disease did not progress but remained localized. When a few drops of a suspension of 5,000,000 bacilli of "R" colony were instilled into the conjunctiva of a normal guinea pig, the result was wholly unlike that observed with the "S" colony. The animal failed to react to tuberculin up to the fourth week and no lesions were observed in the eye. In some animals which were killed for comparison, the only ones found were healed tubercles in the posterior auricular lymph nodes. Intratesticular, intracardiac and intracutaneous inoculations produced lesions which in the majority of instances healed. Lesions were observed in rabbits inoculated by various methods, but they were not like those observed with the "S"

colony. No tubercles were found in the lungs, spleen or liver, up to 8 weeks.

The foregoing observations on guinea pigs and rabbits which were inoculated with the two colonies, were of brief duration (4 months). The results might have been different if we had allowed the animals inoculated with the "R" colony to live longer. We consider this of great importance and believe that some of our animals inoculated with the "R" colony after 12 to 18 months or more might have died of tuberculosis because of a reversion of the bacilli in the animal to the "S" type. This will be referred to in a later paper.

Dissociation of the Avian Tubercle Bacillus A_1

The culture of avian tubercle bacillus studied was A_1, an organism which has been in our possession for many years. It is slightly pathogenic for chickens and only occasionally produces progressive disease in rabbits.

A suspension from a 2 weeks' growth of a plain egg culture was prepared by the usual method and filtered. The filtrate was picked up with a sterile capillary pipette and six drops seeded on the surface of gentian-violet-egg plates. The ordinary gentian-violet-egg medium may be used satisfactorily in dissociating the avian tubercle bacillus, sodium taurocholate not being necessary for it. After several weeks, a number of single colonies appeared on the plate, the majority of which were flat, slightly opaque, raised in the center, and the periphery appeared translucent. Upon careful examination with a low power lens, a small number of somewhat darker, opaque, round colonies could be seen scattered over the surface.

This second colony is like a moth-ball, moist, glistening and easily emulsified in an even suspension, growing profusely in alkaline synthetic medium but without any diffusion of pigment into the fluid. The first colony is flat, spreading and very similar to the "R" colony of the bovine culture. It grows more profusely in synthetic medium with an acid reaction and produces a slight amount of pigment (see Plate 20, 3, 4, 5 and 6).

The two colonies on gentian-violet-egg medium revealed some very striking changes when kept in the incubator for a period of 8 months. The "S" colony first developed into a moth-ball and after several months spread and assumed a flat appearance, remaining flat for another 3 months. After 7 months from the time of seeding from the mother colonies secondary daughter colonies appeared in the form of papillae. They have, at times, a worm-like appearance sprouting in all directions, the growth being sprinkled with a few moth-ball papillae. The periphery of the mother colony is moist, perfectly smooth and cream-like in color. Subcul-

tures from the periphery developed mostly colonies with typical "R" characteristics, while the worm-like growth or the moth-ball papillae developed some "S" colonies (see Plate 20, 1 and 2).

The "S" organism is highly pathogenic for chickens, $\frac{1}{3}$ mg. inoculated intravenously producing septicemia and death in 30 days. The "R" colony is not very virulent, and will not produce the same type of disease.

Dissociation of the Human Tubercle Bacillus

An attempt was made to dissociate a human type of tubercle bacillus known as H_{37}. In the preceding pages we have mentioned that this organism was formerly highly pathogenic for guinea pigs, but that in the last 3 or 4 years in some instances it has failed to produce progressive disease. Such fluctuations of virulence appear to be connected with the media used.

It was noted that the diminution of virulence occurred when the organism was cultivated on veal broth with an acid reaction. On the other hand, if cultivated on Proskauer and Beck synthetic medium which is slightly buffered and has an alkaline reaction, no loss of virulence was observed. Ten bacilli from such a culture very often produced tuberculosis in guinea pigs, leading to death in from 100 to 150 days. It was very difficult to dissociate H_{37} by the method used in dissociating the other strains. The difficulty, in all probability, could be explained by the facts (1) that we used a suspension prepared from Proskauer and Beck medium cultures, in which virulent "S" organisms predominate, and (2) that the colonies which appeared on the gentian-violet-egg plates from a filtered suspension, differed decidedly from those seen in bovine, BCG or avian cultures. Each variant developed colonies of different topography, and there was no fixed characteristic which could help us to separate the colonies. They were unstable, and the gentian-violet-taurocholate medium was of no value. Another method had to be devised. Remembering that this organism lost its virulence when cultivated on glycerine-veal broth of acid reaction, we decided to cultivate the organism on Sauton fluid medium buffered at pH 6.6. After a number of cultural passages through this medium, followed by cultivation on gentian-violet-egg plates, we have dissociated two or perhaps three colonies which can be seen in Plate 21, 1, 32, 2, 3, 26 and 4. The supposed "R" colony is very waxy, much raised, with large folds. The periphery is sharply outlined, and emulsifies with difficulty in salt solution. It produces a chromogenic soluble substance in acid synthetic media. When cultivated on solid media, it at first appears creamy-yellow, a characteristic noted of "R" colonies of other bacteria, but as it ages it becomes yellow. The "S" colony is flat, spreading, composed of small wrinkles, appearing at times like ground glass. This colony is

easily emulsified in salt solution of pH 7.2 and grows best on synthetic media of pH 7.6.

The above is the description of the two main colonies. Recently, we have observed other variants which will be described in a subsequent study.

Dissociation of Bacillus Calmette-Guerin (BCG)

The result of dissociation of BCG was published in a recent article (10). At that time the two extreme "R" and "S" colonies were illustrated and fully described.

It has been noted by the workers engaged in the study of this organism that cultures of BCG distributed by the Pasteur Institute several years ago were more virulent for guinea pigs than the recent ones. In a previous study (10) we offered an explanation for the loss of virulence after prolonged cultivation on glycerine-potato-bile medium. We pointed out that only the "R" strain develops readily on that medium and that "S" does not grow well and no visible colonies can be seen in cultures. Therefore, by the process of gradual elimination, the undissociated cultures at present harbor only a very small number of "S" organisms.

Culture BCG 359 (which Petroff obtained in the fall of 1928 from the Pasteur Institute) was more difficult to dissociate than the three cultures which had previously been obtained. Out of some thirty-five plates made from the same suspension, only one plate showed two distinct and one questionable "S" colonies. For this reason we advocate the use of a large number of plates (30 to 50) in attempting to dissociate BCG.

The four following methods have proved successful in our hands in the dissociation of BCG:

1. The original BCG culture was cultivated on Proskauer and Beck's synthetic medium with a reaction of pH 7.4 to 7.6 and subcultures were made every 2 weeks. After six to eight subcultures many "S" colonies could be isolated by the plating method.

2. Rabbits were inoculated intravenously or intraperitoneally four times at intervals of 3 to 4 days with a 5 mg. suspension of undissociated BCG killed in the water bath at 100°C. for ½ hour. 4 weeks from the beginning of the inoculation the rabbits were bled aseptically and the clear serum was added to Proskauer and

Beck's medium in the proportion of 10 per cent. After a preliminary incubation to test sterility, the surface of the flasks was seeded with the original BCG culture and subinoculation made on similar medium every 2 weeks. If a transplant is made from an eight to ten passage on gentian-violet plates, the "S" colony can be separated. At the same time the whole culture acquires virulence for guinea pigs.

3. The virulence of the original culture has been increased, with the "S" colony predominating, by cultivation on synthetic medium containing 10 per cent normal rabbit serum. However, the transformation is much slower in our experience.

4. The "S" colony can be dissociated from abscesses formed in guinea pigs. Guinea pigs were inoculated with 10 mg. of the original BCG culture. 6 to 8 weeks later the same amount was again inoculated subcutaneously. Several weeks after the second inoculation, plate culture prepared from the abscesses revealed a number of "S" colonies.

Dissociation has been accomplished successfully in our hands by any of the four preceding methods. Workers following them will find that single colonies will appear on the plates after from 40 to 60 days. On careful examination, several different colonies with distinct cultural characteristics will be found present in the plates.

We shall describe at first the two widely different colonies which we have studied more extensively than any others.

The "R" colony (see Plate 22, 39) appears waxy, with slightly raised center, sloping gradually towards the periphery. Sometimes the colonies appear in rosette form, but the general characteristic is that of a coil of small intestines. The periphery is clear cut, raised and does not extend into the medium. This colony is at first slightly chromogenic, becomes with age a dark orange, and is very difficult to emulsify in salt solution of pH 7.4. On Sauton's synthetic medium it grows very profusely in small islands. A mass of growth can be lifted very easily without disturbing the surrounding colonies. The organism grows well at pH 6.5 to 6.8. It will grow for the first and second subculturing on alkaline broth for the probable reason that a small amount of the medium is transferred from the original culture; but after the third subculturing the colonies cease to develop.

This colony grows very readily on Calmette glycerol-potato-bile medium. It appears first in small round pebble-like colonies, the surfaces of which are perfectly smooth. They gradually increase in size, occasionally reaching 1 to 2 mm. in diameter. The surface of this colony remains perfectly smooth for the first month. 4 to 6 weeks later a small granular papilla appears at the end, gradually covering the whole surface with secondary growth. At the end of the third month several shoots of coarser papillae develop. Eventually the original colony is covered with this secondary growth, entirely changing its appearance.

The "S" colony is composed of minute irregular wrinkles and the whole structure is much more delicate than the "R" colony. It appears very much like honey-

comb. The periphery is veil-like and extends into the medium, will not grow on glycerol-potato-bile, grows best on Proskauer and Beck medium pH 7.8, is easily emulsified and is more sensitive to gentian-violet-egg medium (see Plate 22, 40).

The pathogenicity of these two colonies has been described in our recent paper (10) and to avoid repetition we have omitted the experimental data on animals.

Reversibility of "R" to "S" Virulent Organism

Recently, we (10) have described in detail the method used in changing the "R" into "S" colonies. Since that time we have repeated the experiments sufficiently often to say here that when the "R" colony is cultivated on media containing anti-"R" serum after the 8th to 10th cultural passages, the offspring of the original colony becomes "S."

SUMMARY

The recent advances in the study of the other bacteria with application to the dissociation phenomenon, have been applied in the study of acid-fast organisms. For some time, we have realized that the term "dissociation" as employed at present, is not adequate to explain the instability and subsequent variation which occur in cultures. But for uniformity of bacteriological nomenclature, we have adopted the term until a better one is coined. In describing the "R" and "S" colonies, we have had to depart somewhat from the general usage of these terms, that is the "R" meaning rough, and "S" smooth. The colonies of acid-fast organisms are relatively varied and complex. It seems better to employ the letter "R" to indicate greater resistance to environment and relative avirulence; and "S" to indicate colonies which are more sensitive to environment while possessing for certain species relatively great virulence. The terms "rough" and "smooth" apply directly only to avian tubercle bacillus, when cultivated on plain gentian-violet-egg medium. The avirulent colony isolated from this culture is flat and somewhat rough in appearance. The virulent is perfectly smooth, round and resembling a moth-ball. The physical properties are different. They have been fully described elsewhere.

When the bovine "R" and "S" are cultivated on plain gentian-violet-egg medium, differentiation is very difficult. At times they are

almost indistinguishable, but the addition of 0.25 per cent sodium taurocholate to the medium, alters completely the topography of the colony. The "S" appears in perfectly round smooth moth-balls, and the "R" in larger, spreading and somewhat rough colonies.

Lacking suitable media, the human tubercle bacillus H_{37} has been more difficult to dissociate. After 2 years' study, using various media, we have been able to dissociate two types of colonies; but as the animal experiments are not yet completed, very little more than that can be said at present.

We have dissociated two extreme types of colonies from four BCG cultures obtained from various sources. Each of these four cultures has revealed the same types of colonies. For details the reader is referred to a recent paper (10). In this publication we have included photographs taken from time to time in order to keep a record of our observations. When studying the photographs, the reader will notice considerable variation in some of the colonies. Unquestionably, there are more than two types of colonies developing during the life cycle of the organism, but at present we have considered and confined ourselves to only the two extreme types, one which can produce progressive disease, leading to the death of the animal, and the other which is but slightly virulent, and sometimes not at all so for susceptible animals.

Full details of the technique employed by us have been described in the test. Anyone attempting to duplicate the work must strictly adhere to the technique described. *Departures from it may lead to failure.*

The underlying factors favoring dissociation are not yet clearly understood. We believe that every single bacillus contains the two components, "R" and "S." If the environment is favorable for the development of the "R" component, the offspring will be "R's," although the original organism may be "S." Conversely, if the environment is favorable for the "S" and not for the "R" component the "S" will develop. For example, if an avirulent "R" colony obtained from the avian bacilli is cultivated on egg medium, which is favorable for the organism, the offspring after a suitable length of time will develop "S" colonies.

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EXPLANATION OF PLATES

PLATE 18

A, process used in preparation of the rubber bands. a, properly cut rubber band 1½ inches wide. c, front view of rubber band stretched over the wooden disc. d, same, back view. e, number of such discs with stretched bands are strung on iron rod, tightened and autoclaved for 1 hour at 121.3°C. b, properly shaped rubber band.

B, process used in the preparation of suspension of organism for seeding. a, funnels. b, the organism is triturated just above the liquid level. c, Petri dish with the rubber band properly applied.

For details, the reader is referred to the text.

PLATE 19

Colonies of bovine tubercle bacilli B_1. 1, 2, 34 and 38 are on 0.25 per cent taurocholate gentian-violet-egg medium plates, 37 and 3, without taurocholate, ⅞ normal size.

1, the moth balls are "S," the flat are "R" colonies, age 150 days. 38, "S" colony, age 298 days, secondary variants have developed. 34, typical "R" colony, age 68 days. 2, typical "S" colony, age 68 days. 37 and 3, are "R" and "S" colonies cultivated on medium from which the taurocholate has been omitted; differentiation is very difficult.

PLATE 20

Colonies of avian tubercle bacilli A_1, on gentian-violet-egg medium plates; 1, 3, 4, ⅞ normal size; 2, 5, 6, magnification 7 ×.

1, "S" colony, secondary growth and papillae, age 286 days. 2, same as (1), magnified 7 ×. 3, typical "S" colony, a subculture from the papillae, 1-a, age 40

days. 4, typical "R" colony, a subculture from the periphery of 1-b, age 40 days. 5, higher magnification of (3). 6, higher magnification of (4).

PLATE 21

Colonies of human tubercle bacilli H_{37} on gentian-violet-egg medium plates, $\frac{7}{8}$ normal size.

1, undissociated mostly "S," few typical "R" colony structure has developed after 3 months, age 168 days. 32, ? "R" colony with autolyzed center, age 86 days. 2 and 26, "R" colonies, age, former 168, latter 100 days. 3, "S" colony which developed "R" in the center periphery is "S," age 86 days. 4, typical "S" colony, age 86 days.

PLATE 22

Colonies of BCG on gentian-violet-egg medium plates, $\frac{7}{8}$ normal size.

39, Strain 3 "R" colony, age 67 days. 40, Strain 3 "S" colony, age 86 days. 1, Strain "Park," secondary veil-like growth (a) can be seen shooting under the mother colony. Subcultures from such growth developed "S" like structure, age 242 days. 4, Strain 3 undissociated. The structure is similar to (1), the secondary growth can be seen in (b), age 210 days. 3, Strain "Park," probably intermediate colony, age 242 days. 14, Strain 3 undissociated. This is very similar to (3), age 210 days.

The original cultures, dissociated and illustrated in Plates 22, 23, 24, and 25 were obtained from the following sources. No. 3, Pasteur Institute strain obtained in the spring of 1927. No. 359, Pasteur Institute strain, given in person to one of the authors in the fall of 1928. No. "Park" strain, a culture sent to Dr. Baldwin by Dr. W. H. Park, New York City.

PLATE 23

Colonies of BCG on gentian-violet-egg medium plates, $\frac{7}{8}$ normal size.

20, subculture from undissociated Strain 3 organisms which has been cultivated through 7 passages on synthetic medium containing 10 per cent normal sera. Majority of the colonies are "S." Few of them have developed in the center an "R" structure, age 283 days. 21, Strain 359, at the beginning these were typical "S," as they aged "R" structure developed in the center, periphery continues to be "S," age 215 days. 22, this is unexplainable at present, some parts are "S" and the others look like "R," age 110 days. 9, Strain 359, undissociated, secondary growth can be seen developing from the original colonies, age 240 days. 16, Strain 3 undissociated, consisting of "R," "S" and intermediate, age 240 days. 5, Strain 359 undissociated, the growth is moist with autolyzed zone, age 110 days.

PLATE 24

Colonies of BCG on gentian-violet-egg medium plates, $\frac{7}{8}$ normal size.

12, Strain "Park" S?, colony, age 130 days. 2, Strain 359, undissociated secondary veil-like growth, a and b. 18, Strain 3 undissociated; this plate was

seeded from a synthetic anti-sera medium culture. Majority of colonies on this plate are "S," age 210 days. 6, Strain 359. This colony erroneously has been described by Kraus, Tzekhnovitzer and Zeyland as "S" colony. Although it is smooth in structure, it is not the true "S" colony which we have described in the text, age 110 days. 17, Strain 3 "S" colony which has been cultivated for a long period on acid medium. The organisms after such treatment have mutated and changed into "R," age 110 days. 13, Strain "Park" "S" colony, age 110 days.

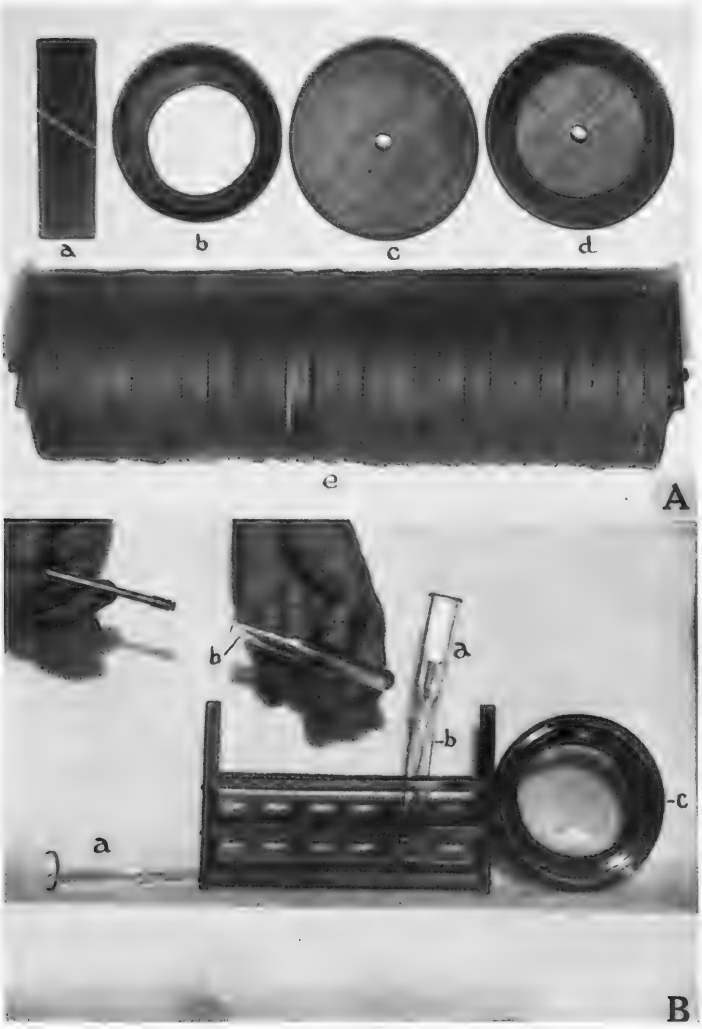
PLATE 25

Colonies of BCG on gentian-violet-egg medium plates, $\frac{1}{3}$ normal size.

10, Strain 3 "S" colony which first developed "S" structure and as aged developed "R" structure in the center, age 281 days. 19, Strain 3 undissociated. It is a subculture from anti-sera growth; periphery "S" and the center is "R" structure, age 281 days. 11, Strain 3 "S" colony. Three zones can be seen, the center is "R," periphery is "S," the intermediate zone is undetermined at present, age 154 days. 7, Strain 3 undissociated organisms, giving same characteristics as (11), age 156 days. 8, Strain 359, colonies on sodium taurocholate medium, age 150 days. 4, Strain "Park." Note the secondary veil-like growth (a) shooting under the mother colony. Such growth upon subculturing develops many "S" colonies, age 210 days.

PLATE 26

Cultures of tubercle bacilli isolated from six patients, directly on gentian-violet-egg medium plates, using sodium hydrate digestion. Every one of these cultures revealed two types of colonies. Magnification 7 ×.



(Petroff and Steenken: Tubercle bacillus. I)

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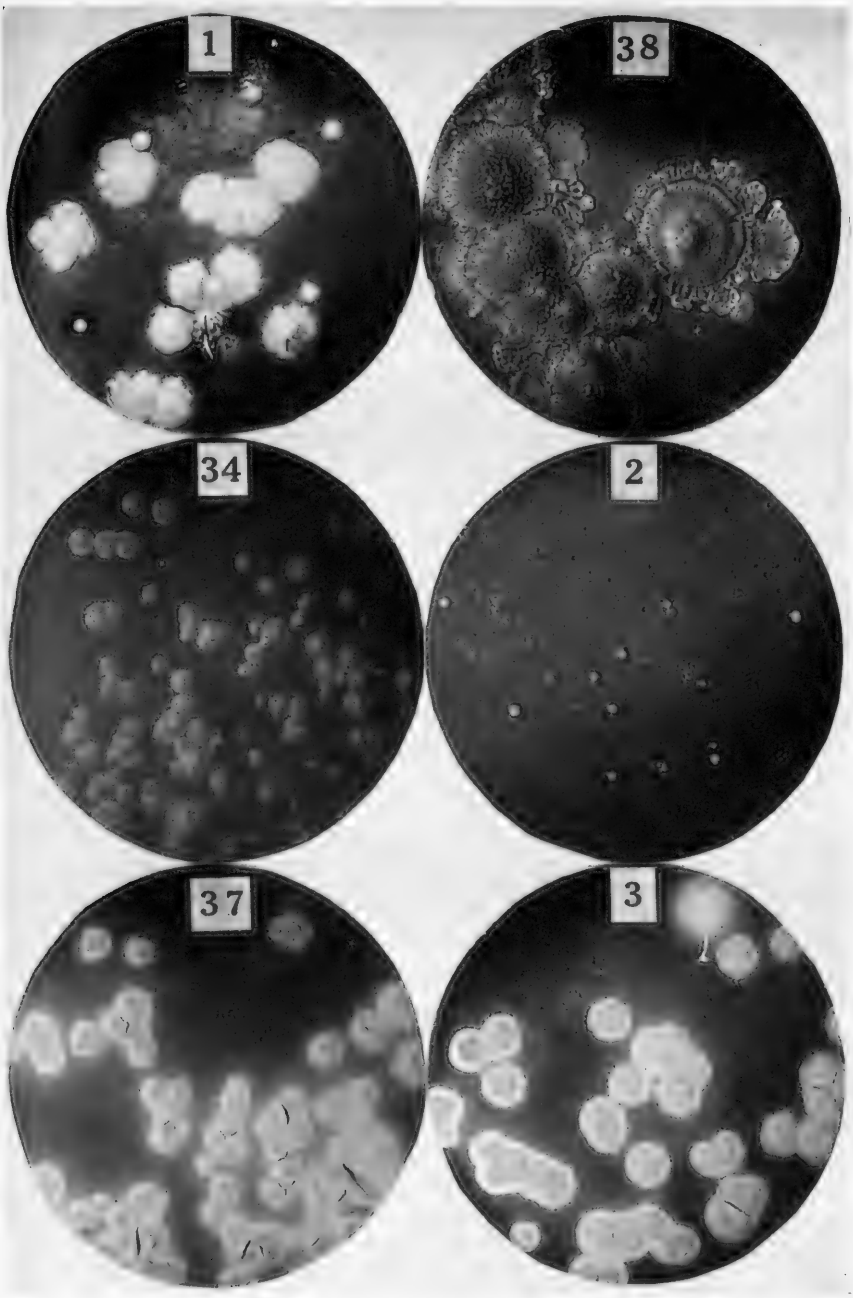

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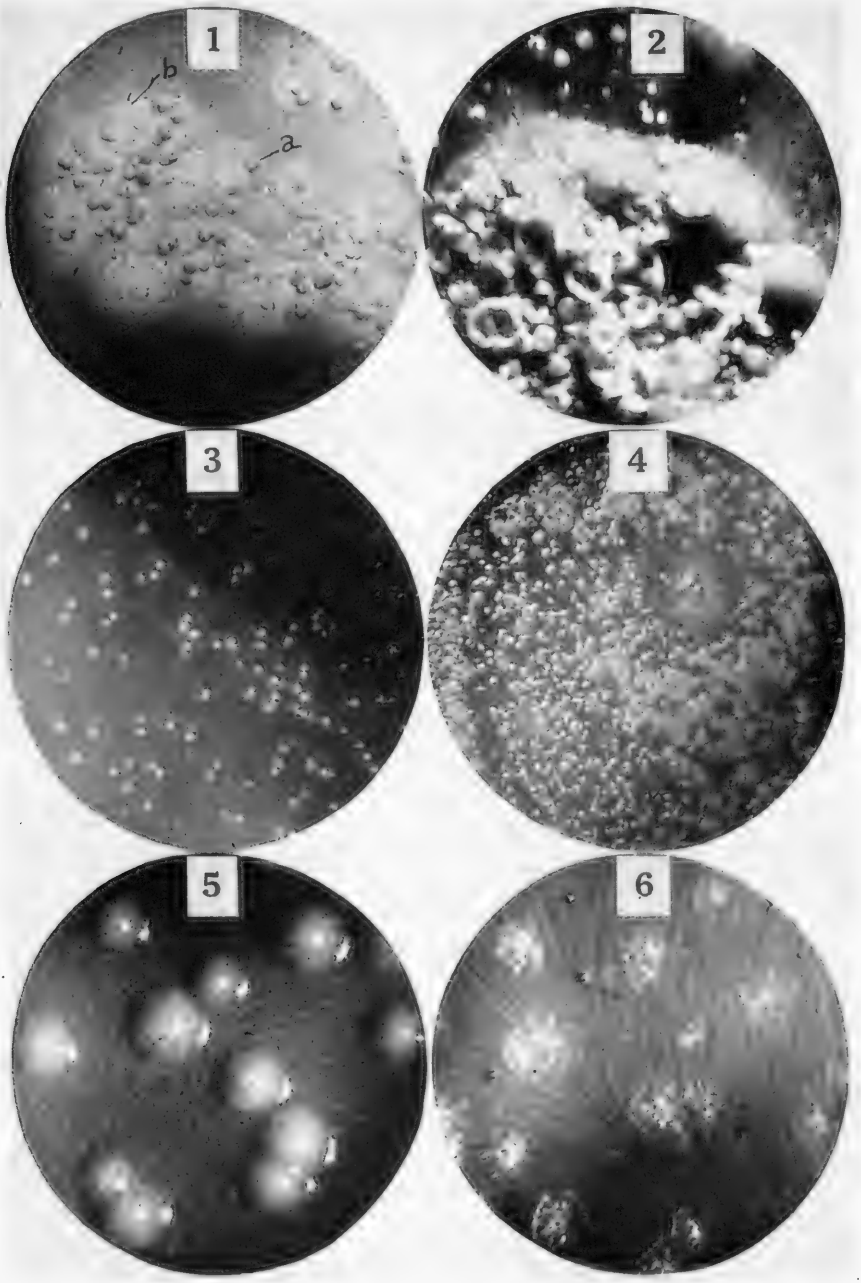

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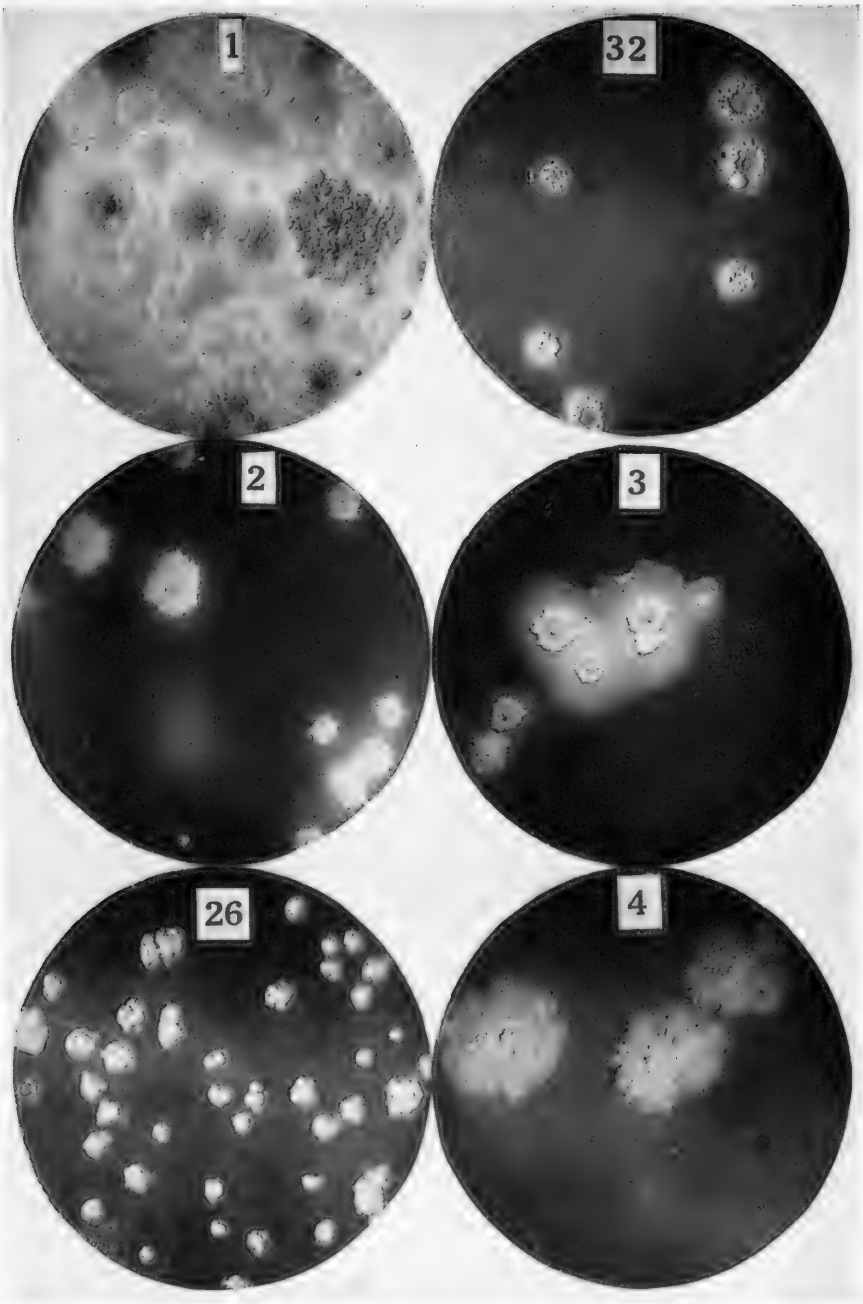

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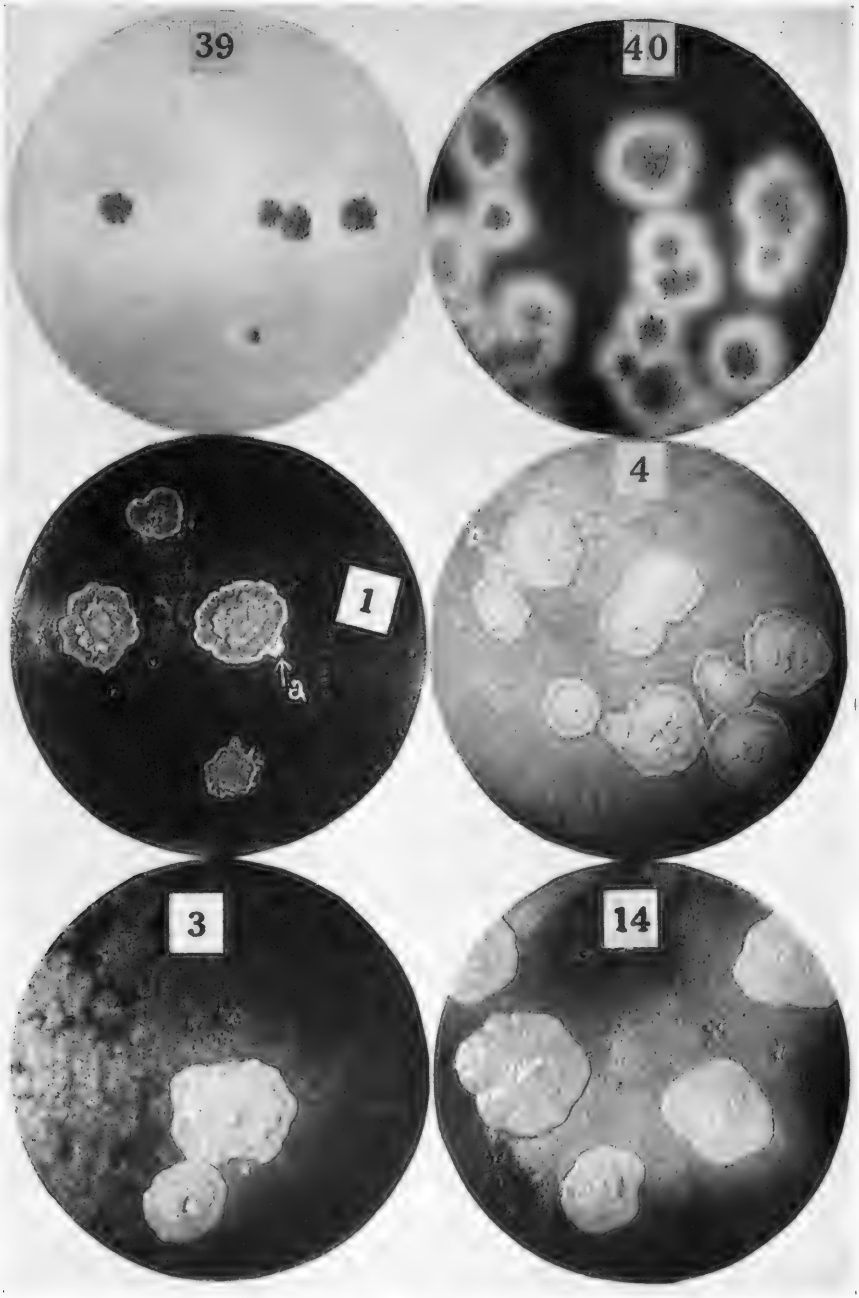

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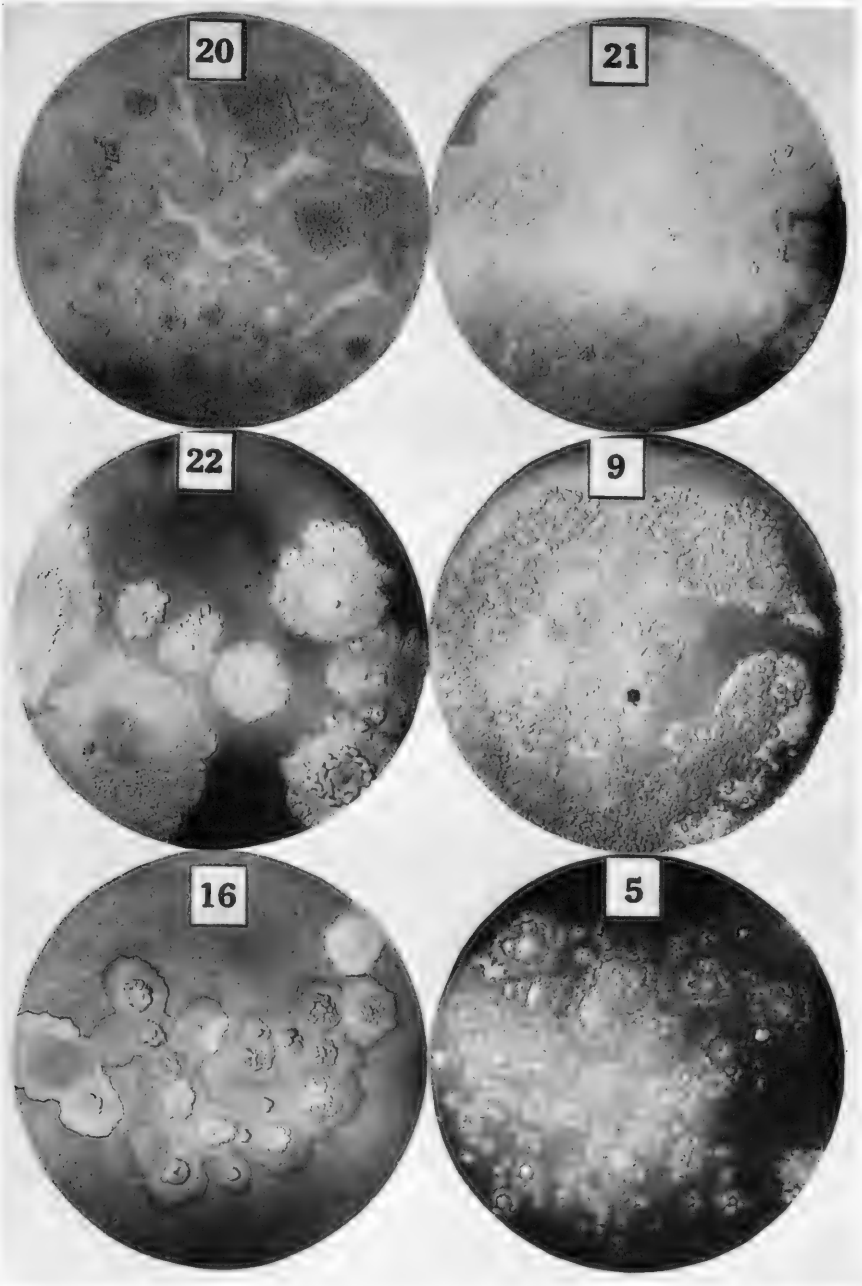
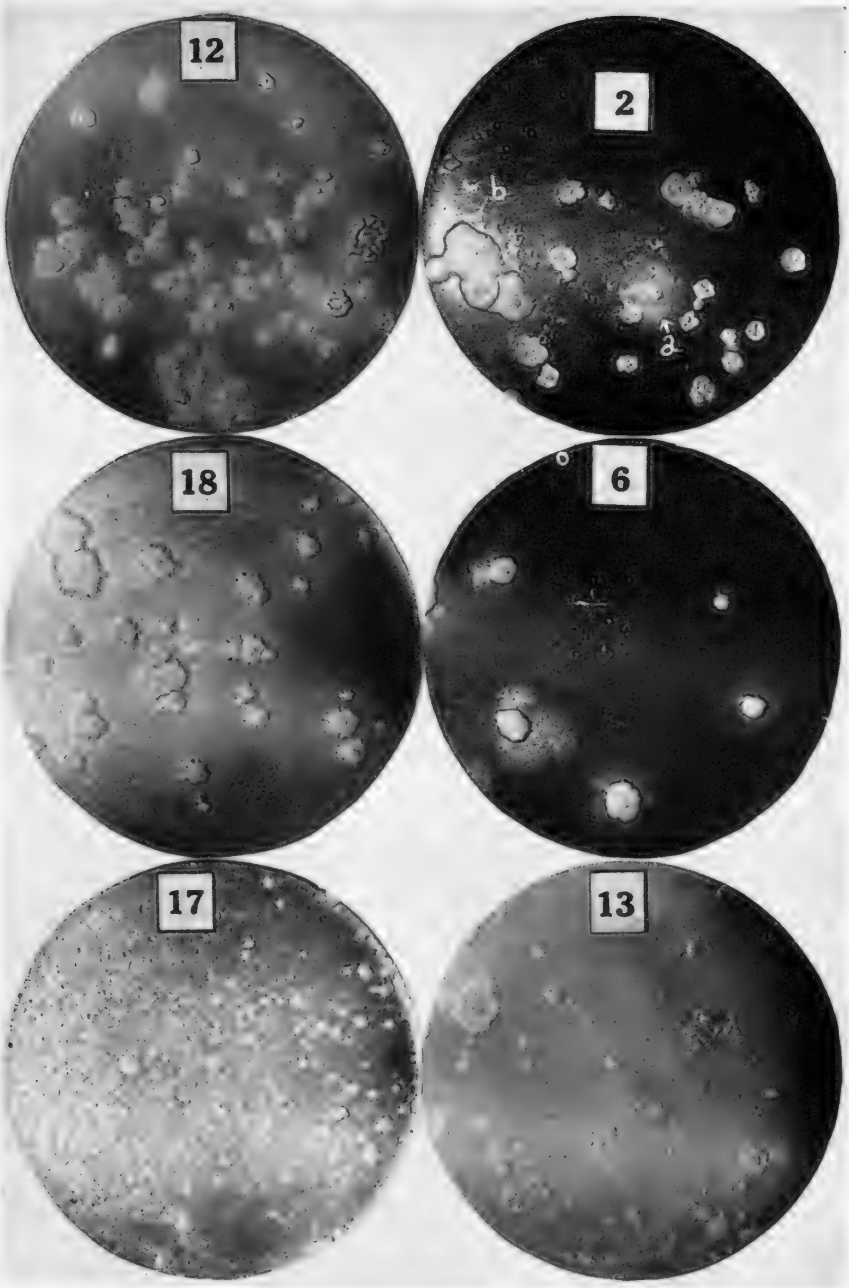


(Petroff and Steenken: Tubercle bacillus. I)



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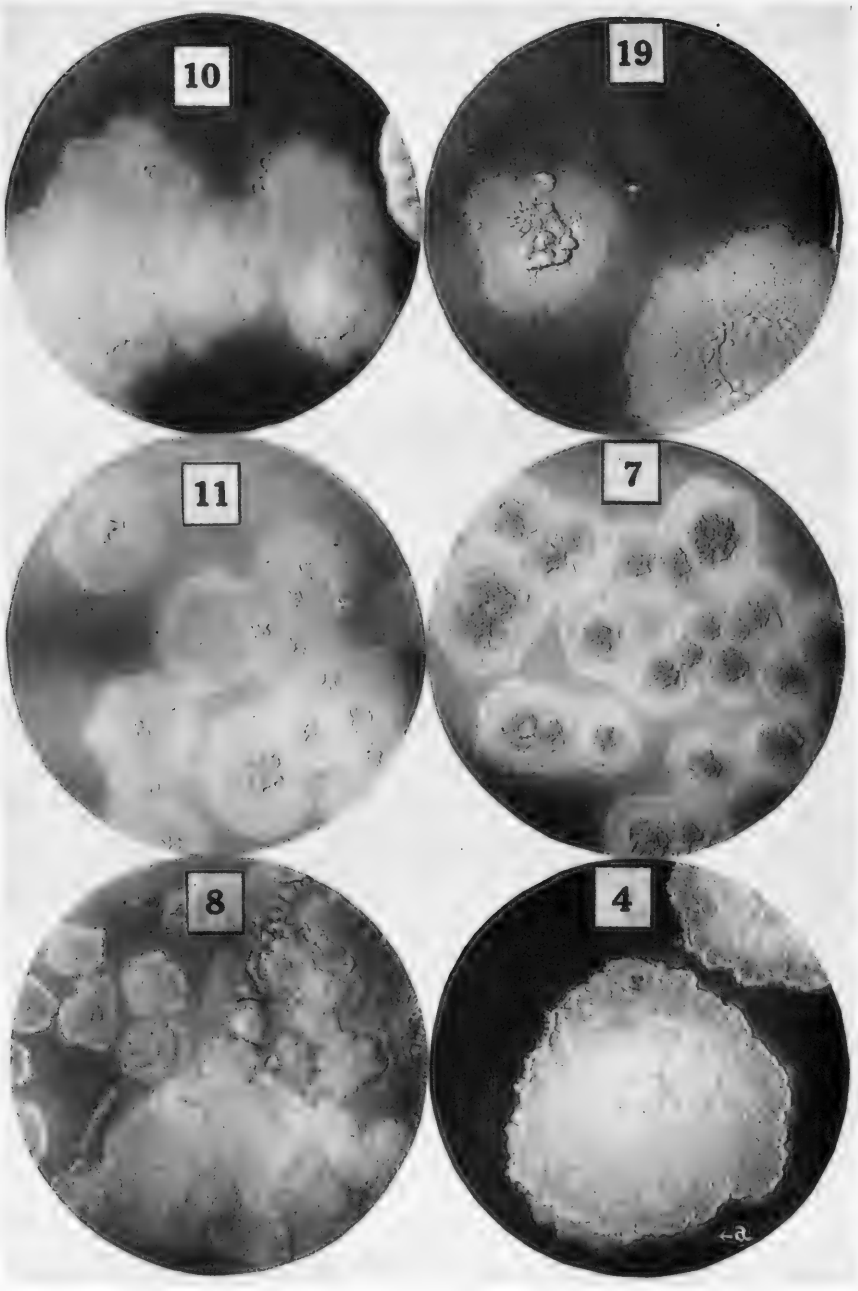

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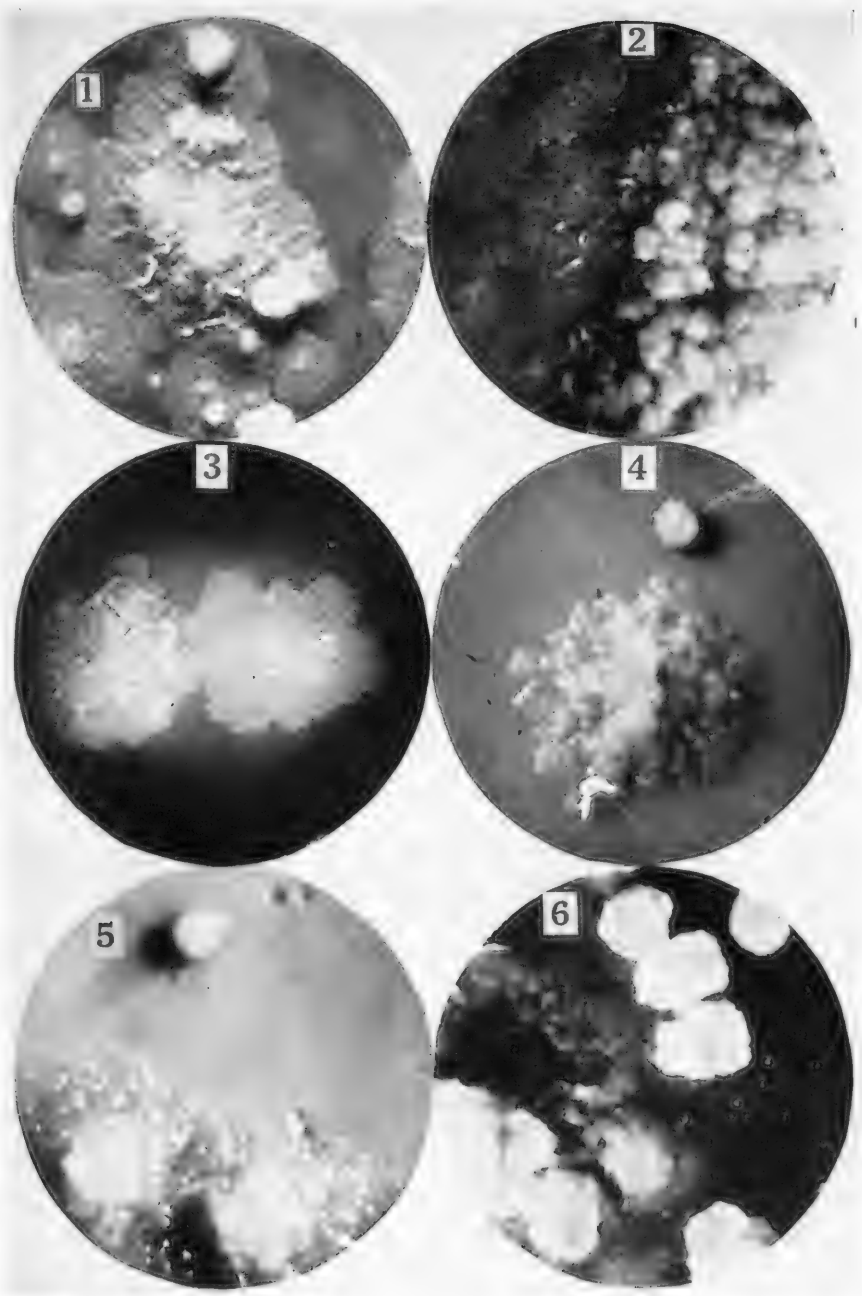

(Petroff and Steenken: Tubercle bacillus. I)

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STUDIES ON MEXICAN TYPHUS FEVER. I

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PLATE 27

(Received for publication, March 10, 1930)

The following notes on experiments with the virus of typhus fever are presented in the briefest possible manner, since to a considerable extent they represent confirmations of previous work. Our results are published because, in a subject as difficult as this one, confirmation of important facts that have been observed a few times only and have in some cases been questioned, constitutes a necessary criterion for the planning of further investigation.

The Filtration of Typhus Virus

There are irregularities in reports of the filtration of this virus. Negative results have been reported by a number of workers (Ricketts and Wilder (1) Anderson and Goldberger (2), Olitsky (3)). Nicolle, Conor and Conseil (4), however, obtained doubtful results, and Nicolle and Lebailly (5) reported two positive filtrations through Chamberland L_2 filters which held back *Bacillus melitensis*.

We carried out a considerable number of filtration experiments through Berkefeld V candles with guinea pig brain proved infectious by control, using the method worked out and published by Ward and Tang (6), which gives almost 100 per cent filtration with brain material of typical filterable agents such as herpes and with the fresh pulp of vaccinia. This, therefore, established an excellent basis for comparison. *Prodigiosus* was used as control, and control inoculations of unfiltered material were always done. This understood, we omit protocols.

Twelve filtrations of brain material from European typhus gave only one filtration in which controls were satisfactory and the virus passed through. Of five

brain filtrations of Mexican typhus, only one positive filtration was obtained in an otherwise satisfactory experiment.

The brain having given us largely negative results, we passed on to the filtration through Berkefeld V candles of plasma from the defibrinated blood of Mexican typhus guinea pigs which, in the unfiltered state, was infectious in amounts from 0.05 to 0.2 cc. Plasma was ten times diluted with broth and filtered as before with suitable controls. Of ten Mexican typhus plasma filtrations, there were three experiments in which all the criteria of successful filtration with proper control were fulfilled. In all successful filtrations, the typhus nature of the disease was proved by transfer— in some by immunity test and in others by the demonstration of brain lesions.

Berkefeld N candles were used in six filtrations of plasma, these filters letting air through, under water, at pressures of from 10 to 14 pounds, as contrasted with about 6 pounds for the V candles. All these filtrations were negative.

In view of the regularity with which, with our present methods, we have been able to filter typical filterable agents like those of herpes and vaccinia virus, our results with the typhus material attain a particular significance. The comparison with herpes is especially important since, in both herpes and typhus, brain material was used under identical conditions. It would seem from these experiments that the agent of typhus did not, in regard to filtration, fall into the class of the so-called "filterable" agents. Its occasional passage through filters which held back bacteria in our own work as in the isolated experiments of Nicolle and his associates, can be taken to indicate that the typhus agent, while larger than the filterable viruses, yet was smaller than a typical small bacterium—a supposition which would be consistent with the observed size of the Mooser bodies (7) (*Rickettsia prowazeki* seen in the tunica of guinea pigs). Moreover, cultural studies on all the filtrates carried over periods of from 2 to 3 weeks after filtration would indicate that the typhus virus was not bacterial and had no relationship to the occasionally observed diphtheroid organisms that can be cultivated, from time to time, from typhus tunicas. The animals unsuccessfully inoculated with large amounts of filtrate were not immune.

Localization of the Typhus Virus in Guinea Pigs

Consistent with the results of other observers, the virus was found in blood, brain, spleen and scrapings from the tunica vaginalis. In regard to the blood, the cell-free blood plasma was virulent with considerable regularity in amounts of 0.1 cc., and on one occasion titrated down to 0.05 cc.

The question has been raised by Nicolle (8) as to whether the virus is present in the leucocytes. This was examined by producing aleuronat exudates in the peritoneal cavities of guinea pigs at a highly infectious stage of the disease, and washing these as free as possible of red blood cells. Guinea pigs injected with washed leucocytes either developed no temperature whatever or came down very much later than those injected with red cells exposed to the same delays and washing manipulation as the leucocytes from the same animal. Leucocyte injected animals occasionally reacted on the sixteenth or seventeenth day after inoculation, while heart's blood of the same animal gave a typical reaction with testicular swelling on the seventh and eighth days. Guinea pigs inoculated with twice washed red blood cells of the same animal reacted like the whole blood inoculated ones. We conclude from these results that the virus is not normally intra-leucocytic, the occasional reactions being probably due to phagocyted virus.[1]

In attempting to wash red blood cells free of virus, we encountered great difficulty. These experiments were carried out by washing with a sufficient amount of broth so that the last supernatant fluid from the red cell washings represented a dilution, in one case, of 1–7000 of the original plasma. It was apparent from these experiments that the virus was either attached to the red blood cells or actually in them, for the cells remained virulent even when four times washed with 50 cc. of broth.

However, in view of the observations reported with Rocky Mountain spotted fever by Spencer and Parker (9), we carried out an experiment in which normal red cells were exposed to infectious plasma and, after standing for 10 minutes, separated and twice washed with broth sufficient to give a final dilution of the infectious plasma of 1–130. The guinea pig injected with the normal cells so treated came down typically with testicular swelling and a temperature of 105°C. on the ninth day, and the virus could be carried on from this animal in passage.

We conclude from these experiments that in Mexican typhus fever, the virus is not normally present in the circulating leucocytes except perhaps occasionally by phagocytosis; that it is not easily separable from the red cells, though it is probably not within them, but adheres firmly to their surfaces; and that it is present in the plasma in a concentration too low to permit the reasonable hope of seeing *Rickettsia* or Mooser bodies in the plasma preparations.

[1] In experiments going on at present we have been able, by the use of a new stain which has been perfected by our associate Dr. Castaneda and is to be described elsewhere, to observe actual phagocytosis of Mooser bodies in polynuclear neutrophile leucocytes.

Comparative Virulence of Blood Plasma and Tunica Scrapings of the Same Animal

In a number of experiments of this kind we had irregular results, but on two occasions the tunica material was virulent in much smaller amounts than the blood plasma. On one occasion a dilution of 1–1000 of the material from a whole tunica vaginalis, ground in a mortar with broth, produced the typical disease when the plasma of the same guinea pig did not titrate below 0.1 cc. We have never found plasma infectious in amounts lower than 0.05 cc. A peculiarity of this experiment which we cannot explain is the fact that in the case of the tunica just mentioned guinea pigs receiving 0.1 cc., 0.05 cc. and 0.01 cc. remained negative, whereas the one receiving 0.001 cc. came down. We believe that the comparative titrations indicate that the virus is more concentrated in the tunica where the *Rickettsia* bodies are found. (See also Mooser (7).)

Tissue Cultures

Tissue cultures were carried out by the usual manner in guinea pig plasma with tunica cells which had shown the presence of the Mooser bodies. They were incubated at 37.5°, which we believe was a mistake, and the cultures will be repeated at lower temperatures. On the other hand, we were able to observe what was either an increase of the bodies or a plentiful discharge from bursting cells as late as the eleventh day after planting. We were never able to grow these bodies in the second generation, and they disappeared in all tissue cultures when the cells ceased growing.

One week old material from tissue cultures was still infectious. The injection of tissue cultures from which the organisms had disappeared was not infectious.

Our tissue cultures, as far as they have gone, indicate that the *Rickettsia* remain visible only so long as the tissue is alive and growing. The difficulty of transferring to a second generation may be due to the fact that the organisms die out with the cells containing them. Extracellular multiplication could not be proved, because its suggested success—as in the accompanying plate—may easily have been due to a discharge from bursting cells. The tissue cultures seem to indicate again that the observed Mooser bodies or *Rickettsia* are not bacterial in nature, and that the infectiousness of the tissue cultures is roughly parallel with the presence of these appearances.

We were never able to demonstrate the bodies in tissue cultures of spleen or brain.

Intraperitoneal Capsules

Glass capsules, with a hole left in them, were filled with normal plasma and tunica scrapings of infected animals. They were left in the guinea pigs' peritoneal cavities for varying periods. A capsule taken out of the peritoneum of a guinea pig so treated was removed on the sixth day, before the animal had developed any symptoms of disease, and was proved to be still infectious, giving a typical reaction, but no Mooser bodies could be found in smears of the contents. Another guinea pig, into which a similar capsule had been put, showed a temperature of 105° on the twelfth day, but no tunica swelling. The contents of the capsule taken out at this time consisted of clear fluid. There was a fibrinous plug in the mouth of the tube. The smears from these materials were entirely negative for bodies, but the contents injected into another animal gave a typical swelling on the eighth day and a temperature of 105° on the tenth, with typical bodies in the tunica on examination.

Whole infectious blood placed into capsules and similarly observed caused the typical disease, but never showed Mooser bodies when removed and examined.

Our capsule experiments indicate that the virus may remain alive in the peritoneal cavity of a guinea pig in a glass capsule for as long as 12 days, but that the Mooser bodies are too few to be found unless one wishes to assume that all negative investigations of infectious tissues and blood can be explained by the fact that a mutation of form of some kind takes place. This we are loth to assume, largely because of our filtration experiments.

Strains of Mexican Typhus Fever without Swelling of the Testicles

Guinea pigs injected either subcutaneously or intracutaneously do not develop tunica lesions. In such animals a temperature resembling the European typhus temperature develops between the tenth and fifteenth day. If blood from such animals is, at the proper time, injected intraperitoneally into full-grown guinea pigs, these again will often develop swelling. This of course is no proof that we have not carried Mooser bodies along through the negative animals, but it does indicate that the Mooser bodies can remain alive in typical typhus guinea pigs without being detectable in any of the organs, or in the blood, and quite capable of again arousing the characteristic tunica lesion on subsequent proper inoculation.

Whenever, as on three occasions, we have obtained a strain without swelling which has run along for five to seven inoculation generations through adult, intraperitoneally inoculated male guinea pigs, we have found that we were dealing with intercurrent contamination. In our first strain of this kind, which ran for

four generations without swelling, there was no contaminating infection, but the virulence of the strain died down in these animals and was completely gone on the fifth trans-inoculation. The same thing happened in one other strain, and in a third one we found that we were carrying along a low grade bacterial infection, which ended in death in the fourth generation.

In these three cases, the surviving animals were all tested for immunity, and were found to be susceptible.

While such evidence is indirect, it seems to us to support the view that the tunica swelling with Mooser bodies is an integral part of the Mexican typhus infection in guinea pigs, and not an accidental occurrence.

TABLE I

Guinea pig	No. of days between drop in temperature and taking of blood	Protection
1	1	Typical temperature, no tunica lesion*
2	1	Complete protection
3	5	Complete protection
4	5	Incomplete protection typical course**
5	9	Complete protection
6	12	Modified, mild course. No tunica lesion
7	25	No protection
8	34	Typical temperature. No tunica lesion
9	39	No protection

* This serum, taken on the day the temperature dropped, exerted a very definite modification on the course of the fever, which was late in development and never showed orchitis; but the blood of this guinea pig was still virulent, as proved by inoculation of another animal. There was thus the peculiar condition of a blood already containing protective bodies, but still containing virus.

** This serum was kept about 36 hours in the ice chest before use.

Protective Power of Convalescent Serum of Mexican Typhus Guinea Pigs

Table I shows our results.

Two of these guinea pigs, one completely protected by the 1-day serum, the other completely protected by the 5-day serum, were re-inoculated with virus on the thirty-sixth and fortieth day respectively after the primary mixture had been administered. These animals developed no reaction whatever, while controls were characteristic. It appeared as though the mixture had conferred an active immunity.

These experiments indicate that convalescent serum mixed with virus before injection will protect if the serum is taken between the second and the tenth day after defervescence; that after the tenth day such protective action is doubtful, and that after the twentieth day it is negative. The two animals cited also indicate that guinea pigs so treated may be immune for at least 3 weeks, and that probably—since the injection of serum separately from the virus does not protect—they represent an active rather than a passive immunity.

The rapidity with which the protective bodies disappear from the blood would suggest that they are not of the nature of ordinary bacterial antibodies.

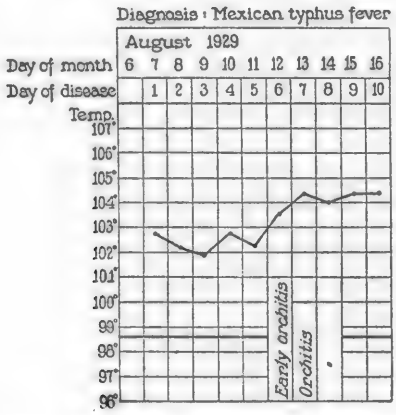

CHART 1.[2] Guinea pig 2–03. Source of virus, simple transfer from 2–02.

Complement fixations were carried out, using virulent plasma as the antigen and protective serum as the antibody, and by such a technique absolutely no indication of complement fixation was obtained.[3]

[2] The word orchitis is used for purposes of convenience; the lesions are almost entirely limited to the tunica and surface of the testicle.

[3] We do not attach much negative value to the complement fixations because of the surely extreme dilution of the antigen in the virulent plasma.

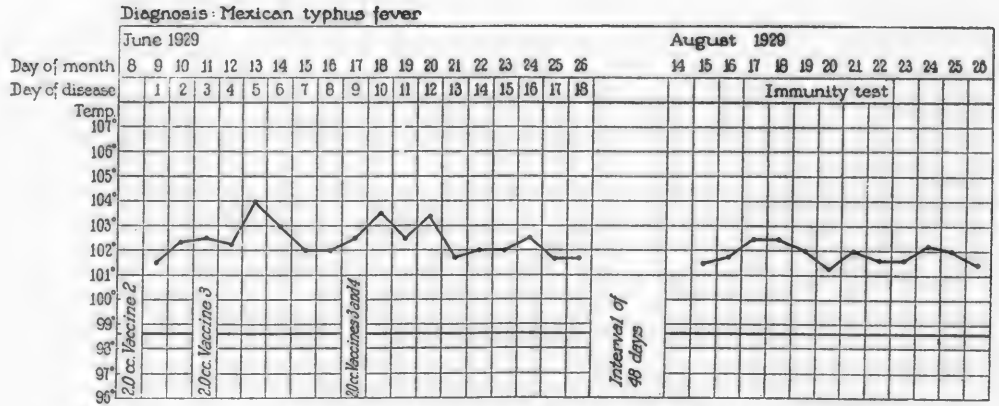

CHART 2. Guinea pig 1–68. Tunic vaccine 2.

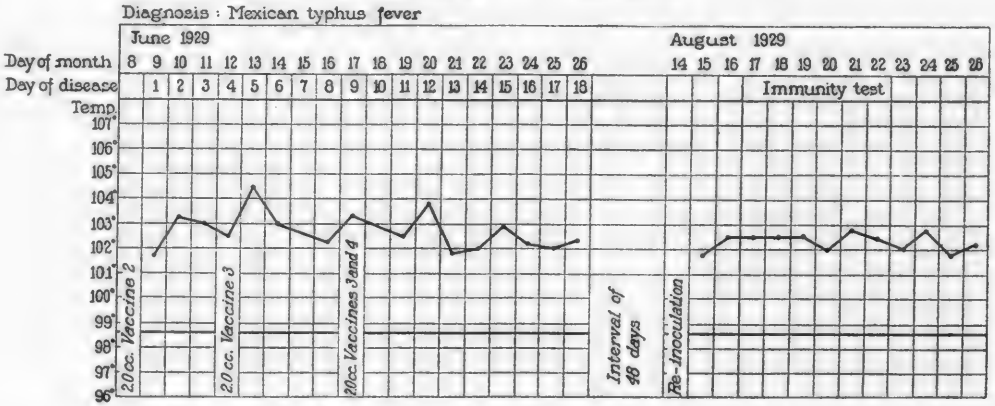

CHART 3. Guinea pig 1–67. Tunic vaccine 2.

Protection of Animals with Formalinized Tunica Material

Both from the experiments of Spencer and Parker (9) and of Conner (10) with spotted fever, it appeared that protection with dead infectious material might be feasible in *Rickettsia* diseases, provided that a sufficient concentration of virus could be attained. Having found that the tunica scrapings, containing many of the Mooser bodies, were much more highly infectious than the blood plasma of the same guinea pig, we thought it advisable to attempt active immunization with such material killed with weak formalin solutions by more or less the same method as that which has been successful for distemper in the hands of Laidlaw and Dunkin (11). Charts 1 to 4 represent a few experiments which gave encouraging

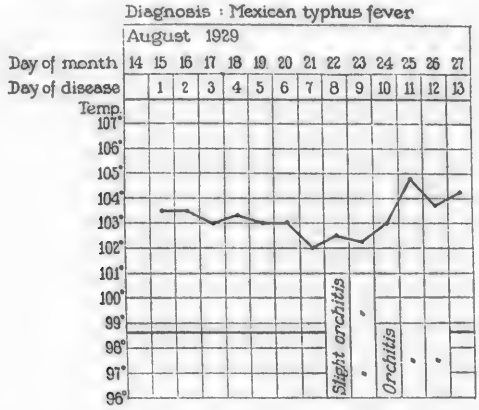

CHART 4. Guinea pig 2–06. Control of reinoculation, simple transfer from 2–03.

results, and which are merely preliminary to a continuation along the same lines with which we are now occupied. The vaccine used was guinea pig tunica taken at a time when it contained Mooser bodies, ground with sand, emulsified in 0.2 per cent formalin, left one night at room temperature and after that put in the ice box. It will be noticed that in the first two animals there was a short rise to 104° after one of the vaccine injections, but that on re-inoculation the animal showed no reaction whatever, although the control came down characteristically.

In the other two, the disease was modified, in that there was swelling of the testes without temperature. This is less significant, because it occasionally—though not often—happens in ordinary Mexican passage animals.

We had one other experiment which was more striking than the first one, but which cannot be included because one of the re-inoculation controls did not react.

Subsequent experiments with formalinized tunics have rendered it uncertain whether we were dealing with dead or attenuated tunic material. This will have to be worked out in the series of experiments with which we are occupied at present, but we believe it worth while to report the above at the present time as an indication that a modification of the disease can be obtained by active immunization with tunic material in which there is a minimum of blood plasma and a maximum of Mooser bodies—a fact which further suggests the etiological importance of these appearances.

Human Infection in the Laboratory

A single case of human infection has occurred in the laboratory. The origin of this case cannot be easily proved, but since the individual was extensively engaged in tunica scraping within the incubation time, and had made only a few blood transfers by heart puncture, which he had been doing for two years without relaxing precautions and without accident, the conclusion is almost forced that this infection originated in tunica material, a small amount of which was probably deposited upon the recently shaven skin of the face.

SUMMARY AND CONCLUSIONS

The preceding studies on typhus fever, chiefly done with a Mexican strain obtained from Dr. Mooser, concern themselves largely with reinvestigations of some of the fundamental problems of this disease.

Filtration experiments carried out with methods almost regularly successful with true filterable viruses, in regard to material, suspension fluid, reaction, nature of filters and pressure employed for filtration, indicate that the virus is not filterable in the ordinary sense in which this expression is employed. It is probably smaller than bacteria and the results of filtration experiments suggest that its magnitude is consistent with the tunica bodies observed by Mooser.

Negative filtrates did not immunize, a result consistent with the previous work of Olitsky.

The virus is present in blood plasma, hardly if at all in leucocytes, and becomes closely associated with the red blood cells, though we do not believe that it is contained in them. It becomes firmly associated

with normal red blood cells when these are exposed to infectious plasma, a result similar to that obtained in Rocky Mountain spotted fever by Spencer and Parker.

In tissue culture, tunica material with Mooser bodies remains alive and virulent for about 10 days, but so far we have not been able to determine that it can keep alive without the presence of living cells. These results do not carry this subject any further than it has been carried for European typhus in tissue cultures with the same method by Wolbach, Schlesinger and Pinkerton (12).

Within glass capsules in the peritoneum of guinea pigs, the virus may remain alive for about the same length of time as in the tissue cultures.

Rough comparative virulence estimations between blood plasma in which it would be hardly possible to find a limited number of Mooser bodies, even though they were present, showed the blood plasma to be less infectious than the tunica material, in which considerable numbers of Mooser bodies were visible.

The testicular swelling characteristic of Mexican typhus and showing the above mentioned bodies—probably *Rickettsia*—may be absent in individual guinea pigs under ordinary conditions and in guinea pigs inoculated by other than the intraperitoneal route. On re-inoculation into the peritoneum after non-orchitic passages, the swelling reappears. Whenever it did not so reappear, we found that the strain had either degenerated in virulence or it had been contaminated by intercurrent infection. Though we can not prove it at the present time, we believe that the tunica lesion is an integral part of this disease in guinea pigs, and not an accidental accompaniment.

Convalescent blood from Mexican typhus guinea pigs mixed in the test tube with virus affords protection if the blood is taken between the first to the tenth day after defervescence. After the third week, the blood no longer contains protective bodies although the guinea pigs may still be immune.

In one case a serum was obtained which was both protective in such a test but at the same time seemed still to contain virus, a result which we cannot explain.

No complement-fixing antibodies were found when virus serum was used as antigen and convalescent serum as antibody. The low concentration of the virus in the serum may account for this.

In a limited number of observations guinea pigs which were negatively inoculated with virus-serum mixtures proved on re-inoculation to be immune. In one of these cases the protective serum mixture with the virus was taken 1 day, in the other 5 days after temperature had returned to normal and the re-inoculations were done 36 and 40 days after the primary injection. This recalls similar experiences of Nicolle and encourages further immunological study in this direction.

In a number of experiments active immunization with formalinized tunica material containing large numbers of the Mooser bodies seems to have modified the course of subsequent inoculations in the direction of protection.

A single accidental human infection seemed particularly associated with tunica material, although this cannot be positively asserted.

All that part of our work which has bearing on the infectious agent is consistent with the assumption that the small, Giemsa-staining bodies observed by Mooser in the tunica of Mexican typhus guinea pigs represent the virus of the disease.

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EXPLANATION OF PLATE 27

FIG. 1. Mooser bodies (*Rickettsia*) in an 8 day tunica tissue culture.

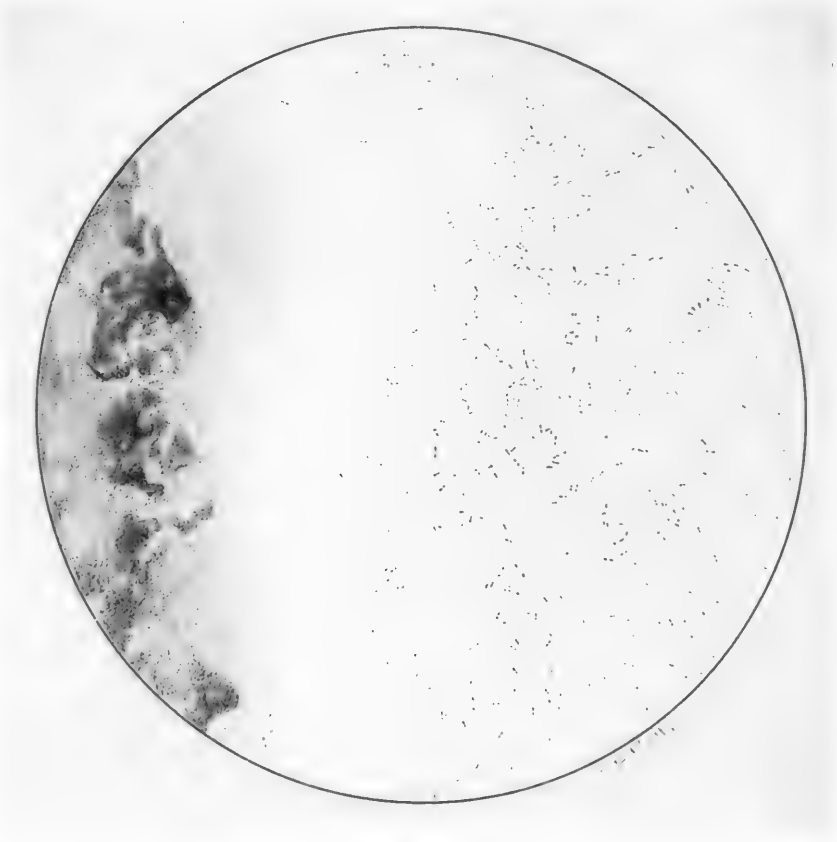

FIG. 1

(Zinsser and Batchelder: Mexican typhus fever. I)

CENTRIFUGE EXPERIMENTS WITH THE VIRUS OF VACCINIA

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The nature of filtrable viruses is still a controversial subject. Gordon (1) suggested that rabbit vaccinia virus is associated in particles big enough to be thrown down by centrifugal force and even thought that the virus might come down under the action of gravity. MacCallum and Oppenheimer (2) stated that vaccine granules can be freed from lymph by differential centrifugation. Bedson (3) supported his theory that the non-passage of herpes virus through a colloidal membrane was due to the size of the virus by the fact that the virus in suspension can be concentrated by high-speed centrifugation. More recently Bland (4) reported similar results in his filtration and centrifuge experiments with vaccinia virus. These results have been criticized, however, on the ground that the experiments were conducted with emulsions or diffusates, where the virus might have been adsorbed by contaminating microorganisms or by cellular structures, and that consequently, when the adsorbing material was centrifuged down, it carried the virus with it.

In a previous communication (5) we have reported that if tissue containing the virus of herpes or vaccinia was suspended in hormone broth instead of saline solution, active filtrates could be constantly obtained. We suggested that if careful centrifuge experiments were carried out with cell-free filtrates, it might be possible to throw some light upon the real nature of the virus. The work recorded here is the result of such an investigation carried out with vaccinia virus.

Preparation and Testing of the Virus Filtrates

Active filtrates of vaccinia virus were prepared according to the method previously described, except that ordinary powdered Japanese glass was used instead

of sand. Fresh virus containing tissue was thoroughly ground in a mortar with the glass powder, and a 5 per cent suspension was made in hormone broth. It was then centrifuged for half an hour at about 2000 r.p.m. and the supernatant fluid poured off for filtration. A Berkefeld V filter was employed after testing its water flow, and a new filter was used for each filtration. The filtration was carried out as soon as possible after the tissue was taken from the animal and always under a negative pressure of 50 cm. of mercury. The filter was further controlled by adding a little of a culture of *B. prodigiosus* to the suspension before filtration was commenced. Big, albino rabbits were used for titrating the activity of the filtrates. The back of the animals was shaved the previous day and the points of injection

TABLE I

Dilution	Unfiltered control			Filtrate		
	3rd day	5th day	7th day	3rd day	5th day	7th day
Undiluted	Not tested	Not tested	Not tested	++	+++	++++
1:10	++	*++++	++++	+	++	+++
1:50	++	++++	++++	−	++	++
1:100	+	++++	++++	−	++	++
1:300	+	++++	++++	−	++	++
1:500	+	++++	++++	−	++	++
1:800	±	++++	++++	−	+	++
1:1000	−	++++	++++	−	−	+
1:1500	−	++++	++++	−	−	−
1:2000	−	+++	+++	−	−	−
1:3000	−	+++	+++	−	−	−
1:5000	−	++	++	−	−	−

* In this and the following tables ++++ represents a maximum reaction and ± a minute papule.

were marked out with a circular ink stamp. The injections were made intradermally, and 0.05 cc. of each dilution was injected. The titration of one such preparation prepared from virus pulp S. M. C., Source 683, is given in Table I.

Centrifuge Experiments

5 or 10 cc. of the filtrate was centrifuged in a conical centrifuge tube in an International size 1, Type S. B. centrifuge for varying lengths of time at the maximum speed—about 4000 r.p.m. Then 0.1 cc. of the fluid was carefully pipetted from the surface and the remaining fluid discarded until a similar amount was left in the bottom of the tube. Dilutions were made in hormone broth of both upper and lower fractions as well as of the uncentrifuged filtrate which served as the control. The dilutions were then injected intradermally into a rabbit. The

result of a 2 hour centrifuge experiment conducted with a green virus filtrate is shown in Table II.

As fresh calf pulp was not always easily obtained, an attempt was made to use rabbits as a source of supply. The preparation of rabbit green virus was very simple; the animals were inoculated on the skin

TABLE II

Dilution	Bottom fluid	Surface fluid	Control
Undiluted	++++	±	++++
1:10	++++	−	+++
1:50	+++	−	++
1:100	++++	−	++
1:300	++	−	++
1:500	+++	−	++
1:800	++	−	++
1:1000	++	−	±
1:1500	++	−	−

In this and the following tables readings were taken on the 5th day, unless stated otherwise.

TABLE III

Dilution	Surface fluid	Bottom fluid
Undiluted	+	++++
1:10	−	++++
1:100	−	++++
1:200	−	+++
1:400	−	+++
1:600	−	++
1:800	−	++
1:1000	−	++
1:1200	−	++

and the virus collected on the fifth day. Table III shows the result of a centrifuge experiment with a filtrate prepared from rabbit pulp. The end-point of the activity of the uncentrifuged filtrate was a dilution of 1:800.

Three strains of neurovaccine derived from intracerebral inoculation of different filtrates were obtained. From these 3 neurotropic strains

a number of active filtrates were prepared. Table IV gives the result of one of the centrifuge experiments made with a filtrate prepared from a neurovaccine brain.

All these experiments show a definite concentration of the virus in the lowermost layer of the centrifuged fluid, leaving the supernatant portion practically inactive. Evidence then seemed to be in favor of the theory that the virus itself or at least its aggregates might really be associated in particles big enough to be spun down by centrifugal force because of the fact that the filtrates contained no microorganisms or other cellular elements upon which the virus might be adsorbed, and so carried down along with this adventitious matter. It has been suggested (6), however, that inert particles present in the filtrates

TABLE IV

Dilution	Surface fluid	Bottom fluid	Control
1:50	−	+++	++
1:100	−	++	++
1:300	−	++	++
1:500	−	++	++
1:800	−	+	+
1:1000	−	++	±

might act as adsorbents, and carry down the virus. In order to meet this criticism, some further experiments were carried out.

The separation of coarser from finer particles by fractional centrifugation is an ordinary laboratory procedure. Since the inert particles assumed to be present in the broth filtrates must be bigger and heavier than the virus, their separation by fractional centrifugation should be possible.

In order to carry out such a separation, more active filtrates are necessary. Such filtrates have been prepared by Ward (7). The only difference in his technique from that already described in this paper is the substitution of pyrex glass powder for ordinary glass powder or sand as a grinding material. Table V gives the titration of a calf green virus filtrate prepared by this modified technique from virus pulp S. M. C., Source 723, and a neurovaccine Filtrate 084 obtained from a rabbit brain originally obtained from vaccine lymph, Lot 97, National Epidemic Prevention Bureau.

TABLE V

Dilution	Green virus 723		Neurovaccine 084	
	Filtrate	Unfiltered	Filtrate	Unfiltered
1:10	+++	++++	++++	++++
1:50	+++	++++	+++	++++
1:100	+++	+++	+++	++++
1:300	+++	+++	+++	++++
1:500	+++	+++	+++	++++
1:800	++	++	+++	+++
1:1000	+	++	++	+++
1:1500	++	++	++	+++
1:3000	++	+++	++	++
1:6000	++	++	++	++
1:12000	++	++	++	++
1:24000	++	++	+	++
1:48000	++	+		
1:120000	±	+		
1:600000		+		
1:1200000		+		

TABLE VI

Dilution	Control	Surface fluid	Bottom fluid
1:10	+++	+++	++++
1:50	++	+++	++++
1:100	++	++	+++
1:300	+++	+++	++++
1:500	+++	++	++++
1:800	++	++	++++
1:1000	++	++	+++
1:1500	++	+	+++
1:3000	+	+	+++
1:6000	+	+	++
1:12000	±	−	++
1:24000	±	−	++
1:48000	−	−	++
1:120000	−	−	++
1:600000	−	−	+
1:1200000	−	−	+

Fractional Centrifuge Experiments

10 cc. of calf green virus Filtrate 723 was centrifuged for 1 hour at 3500 r.p.m. in a centrifuge tube stoppered with a rubber cap. Then 5 cc. from the surface was carefully pipetted off without disturbing the lower portion and transferred to a second tube which was centrifuged for 4 hours at the same speed. Surface and bottom specimens were collected and then the various layers were mixed and a third specimen taken to serve as control. All 3 specimens were then titrated on the same rabbit. The readings were taken on the third day. Table VI shows the result of this titration.

Another experiment was then done in which Filtrate 723 was centrifuged for 4 hours at 3500 r.p.m., the upper half of the fluid removed and placed in the refrigerator over night. Next morning this was centrifuged for 4 hours at the same speed. Table VII shows the result of the titration of the upper and lower layers as well as of the whole fluid as control. Readings were taken on the third day.

TABLE VII

Dilution	Surface fluid	Bottom fluid	Control
1:10	+	++	++
1:50	−	++	+
1:100	−	++	+
1:300	−	++	±
1:500	−	+	+
1:800	−	+	−
1:1000	−	+	−
1:1500	−	+	−
1:3000	−	−	−
1:6000	−	−	−

From the results of Tables VI and VII, especially from Table VII, it seems improbable that the concentration of the virus was due to the adsorption of the virus by the inert particles in the filtrates, because such particles must have been thrown down by the preliminary centrifugation. The results shown in Table VII were confirmed by several other experiments in which the first centrifugation was carried out at 3500 r.p.m. for 4 hours.

DISCUSSION

The ease of filtration of vaccine virus through diatomaceous filters has been demonstrated once more. The success of filtration depends

of course upon many conditions, but the material used for grinding is evidently an important one. Ward's observation (7) that pyrex glass powder serves this purpose better than anything else has been confirmed, and it has been noted that ordinary glass powder is no better than sand. The superiority of pyrex fragments for this purpose is probably due to their not adsorbing the virus rather than to their sharper edges.

Another important condition necessary is the use of very fresh tissue containing the virus. Filtrates prepared from tissue removed from the animal for some time before being filtered are often quite inactive or only slightly active. It is possible that the size of the virus may be altered somehow by some unknown change taking place in the cellular substance of the tissue after death. Ward (7) has shown that if oxygen is excluded from the tissue after removal from the animal, very active filtrates can be obtained after some days, whereas the filtrates of tissue exposed to the air for the same length of time are inactive.

With regard to the success of the centrifuge experiments in concentrating the virus, in the author's opinion it is due to the corpuscular nature of the virus or its aggregates rather than to the presence of inert particles adsorbing and carrying down the virus, for these particles should have been removed by the prolonged preliminary centrifugation.

SUMMARY

Centrifuge experiments have been carried out with cell-free, active filtrates of vaccinia virus. The experiments have shown that the virus can be concentrated by this method, even in filtrates which have been subjected to prolonged preliminary centrifugation to throw down any inert particles which may have been present in the original filtrate. This fact, together with the knowledge that the virus can be almost completely held back by the Berkefeld N filter, as reported previously (5) indicates that the virus may be of considerable size.

The writer wishes to express his gratitude to Dr. R. C. Robertson of the Health Department of Shanghai Municipal Council for many specimens of fresh virus pulp.

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TOTAL WATER AND CHLORIDE CONTENT OF DEHYDRATED RATS

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INTRODUCTION

Dehydration of the body as produced experimentally by placing an obstruction at the pylorus has been extensively studied from the point of view of the changes in the composition and physical properties of the blood caused by the continued loss into the stomach of water and materials; chiefly sodium and, in larger quantity, chloride ion (1, 2, 3, 4). As regards the actual extent of withdrawal of water and of these substances, Gamble and McIver have shown that in rabbits several times the initial blood plasma content of water, of sodium and of chloride ion enters the stomach during the survival period following an experimental obstruction at the pylorus (5). It is thus evident that stores of water and materials behind the vascular system are extensively drawn upon in support of blood plasma volume and composition. The purpose of the study, the results of which are here presented, was to measure the degree of depletion of the water and chloride ion content of the body as a whole which may result from upper intestinal obstruction. It would have been desirable to have determined also the amount of sodium remaining in the body but this was not attempted. Water and chloride ion were measured in the skin of the animal and in the remainder of the carcass, after removing the gastro-intestinal tract, by the method described below. An essential point regarding these measurements is that they were obtained by drying and then digesting the entire skin or carcass, that is, the material was used *in toto*. In some instances the animal was exsanguinated as completely as possible and the water and chloride ion content of the blood determined.

The initial experimental procedure was obstruction of the pylorus by ligature. The resultant continued loss of gastric secretions will

obviously cause body fluid depletion. On reflection, however, it is apparent that the situation contains other factors which may also be expected to cause reduction of the total water content of the body. Pyloric obstruction produces, besides a loss of gastric secretions, the circumstances of fasting and of complete water deprivation. As a result of fasting there must occur consumption of body fat, glycogen and, to some extent, of body protoplasm, with release of constituent water. It would also seem probable that the circumstance of water deprivation will tend to cause subsidence of the water content of the body. With the purpose of appraising together these two additional factors of water removal which are incidentally produced by upper intestinal obstruction, measurements of the loss of water and of chloride ion by unoperated animals subjected to a period of fasting and water deprivation were obtained.

Another item in the plan of study was comparison of the loss of water and chloride ion by rats fasted and deprived of water with the losses found for rats following an experimental obstruction placed at the lower end of the small intestine, water and food being again withheld. The purpose here was to test the expectation that lower intestinal obstruction, in contrast with pyloric obstruction, is a small or perhaps a negligible cause of dehydration, because of a probably more or less complete reabsorption of gastro-intestinal secretions by the small intestine.

Procedures

Operative.—The animals used for these experiments were young adult white rats. Under ether anesthesia the abdomen was opened and obstruction established at the pylorus or just above the appendix by tying with strong silk thread. The incision was closed and covered with collodion. The rats recovered very quickly from the operation. After the desired interval they were killed by ether, the skin was rapidly removed and the gastro-intestinal tract, clamped at both ends, was dissected from the remainder of the carcass. Skin and carcass were then placed in the weighed flasks of the digestion apparatus. When blood was withdrawn this was done under ether anesthesia by opening the thorax with a U-shaped incision after reflecting the skin and delivering the heart through this incision into the mouth of a weighing bottle and then opening the ventricles by snips with a small scissors.

Analytical.—The flask containing the fresh material was weighed and then placed in an electric oven at 100°C. until a constant dried weight was obtained.

A measured excess of N/20 silver nitrate solution was then placed in the flask, several drops of caprylic alcohol added and the reflux condenser fitted on, the joint being of ground glass. 100 cc. of nitric acid was then added through the condenser tube and the mixture cautiously heated over an electric plate until foaming had ceased. The electric heater was then turned full on. Occasionally caprylic alcohol was required during the process of digestion. After a period of 1 or 2 hours, digestion was complete except for tissue fat which was found to have separated completely with the formation of large globules on the top of the mixture. The condenser tube was then rinsed, the flask stoppered and placed in the ice box until the fat solidified. The mixture was then filtered into a volumetric flask, the original flask and precipitate being thoroughly washed with distilled water. The filtrate was then made up to volume. Four 10 cc. samples of the filtrate were pipetted into large pyrex test tubes and titration carried out with N/40 ammonium thiocyanate. One sample was titrated to the first visible end point and another was not carried to the end point. These two tubes were used as comparators in titrating the two remaining samples. Titration figures checked very accurately. Calculation: cc. N/20 silver nitrate added originally less one-half of the amount of N/40 thiocyanate required divided by 20 gives milli-equivalents of chloride ion in the sample.

RESULTS

The data obtained from a control rat, from a rat deprived of food and water for 18 hours, and from three rats sacrified 12 hours after obstructing the pylorus, are presented in Table 1. It should be noted that the average weight of the obstructed rats (192 gm.) is a bit greater than the weight of the control animal (186 gm.) and that in the food and water deprivation experiment the period was 18 hours whereas the interval following pyloric obstruction was 12 hours. The longer period of fasting and water deprivation was used on the basis of an incorrect expectation that these circumstances would be relatively small factors of dehydration. The survival period following pyloric obstruction was found by preliminary experimentation to be between 30 and 40 hours, so that the data in the table describe changes occurring during the first third of the survival period. This short interval was chosen because it had been found that after 12 hours there occurred loss of stomach contents by vomiting or regurgitation and it was desired to compare the amount of chloride ion found in the stomach with the measurement of loss from the body. As regards the total quantity of blood collected by the technic described above, we have considerable doubt that it dependably measures blood volume. In the case of the

obstructed rats, there was evident increase in viscosity which very probably interfered with complete collection. The data are included in the table in order to complete the total values for body weight, water and chloride. The measurements of water per cent and of chloride ion concentration are, of course, not disturbed by the probable error in volume.

In spite of these appreciable defects in the plan of study, comparison of the several sets of data in the table produces certain quite evident

Showing Losses of Water and of Chloride Ion Produced

Losses are estimated by subtracting the values found from those established by the control skin and the gastro-intestinal tract. The "Total" values are the sum of those found for skin, t

Experimental procedure	Weight		Skin						
	Initial	Final	Wt.	H_2O	H_2O	Cl	(Cl)	Wt.	H
	gm.	*gm.*	*gm.*	*gm.*	*per cent*	*m-eq.*	*M*	*gm.*	*g*
Control	186		33.9	18.6	54.9	2.26	.121	129	88.
Fasting and water deprivation 18 hrs.	185	172	28.3	15.9	56.2	1.89	.119	119	81.
Loss, absolute		13	5.6	2.7		.37		10	7.
Loss, per cent		7%	16.5%	14.5%		16.4%		7.8%	8.
Pyloric obstruction 12 hrs.	191	180	28.1	13.9	49.5	1.48	.107	117	77.
	192	180	26.1	14.3	54.8	1.46	.102	120	82.
	193	182	27.3	14.2	52.0	1.51	.106	121	81.
Averages	192	181	27.2	14.1	52.0	1.48	.105	119	80.
Loss, absolute		11	6.7	4.5		.78		10	8.
Loss, per cent		6%	19.7%	24.2%		34.5%		7.8%	9.

findings which may be briefly discussed. To consider first the effects of fasting and water deprivation: we note that the 18 hour period reduced the weight of the animal from 185 gm. to 172 gm. As regards the composition of this loss in terms of water and solids, information is provided by comparing the "total" values for the control and for the fasted animal obtained by adding together the measurements for skin, tissues and blood. A closely parallel loss of water and of solids is indicated by the fact that the values for body weight loss and for reduc-

tion of water content are 8.4 per cent and 8.1 per cent respectively. The loss of weight is thus, as would be expected, the result of consumption of body substance *in toto* rather than of dehydration. Taking the skin by itself, however, and comparing the per cent loss of weight and of water it is unexpectedly evident that a larger loss of solids than of water has occurred producing an appreciable increase of water content. In the remainder of the carcass, designated "tissues" in the table, the loss of water slightly exceeds the loss of solids. The total

ng and Water Deprivation, and (2) by Pyloric Obstruction

The material designated "Tissues" was the remainder of the carcass after removing blood, blood.

		Blood					Total					Gastric contents	
Cl	(Cl)	Wt.	H_2O	H_2O	Cl	(Cl)	Wt.	H_2O	H_2O	Cl	(Cl)	H_2O	Cl
m-eq.	*M*	*gm.*	*gm.*	*per cent*	*m-eq.*	*M*	*gm.*	*gm.*	*per cent*	*m-eq.*		*gm.*	*m-eq.*
4.42	.050	4.52	3.64	80.6	.347	.095	167	111	66.4	7.03	.064	0	0
3.73	.046	5.90	4.60	78.0	.437	.095	153	102	66.7	6.06	.060	0	0
.69							14	9		.97			
15.6%							8.4%	8.1%		13.8%			
3.25	.042	3.54	2.68	75.7	.221	.082	149	95	63.8	4.95	.052	15.9	2.32
3.63	.044	2.53	1.96	77.5	.175	.089	149	99	66.4	5.27	.053	15.0	2.27
3.28	.040	2.06	1.51	73.3	.122	.081	150	97	64.7	4.91	.051	15.9	2.36
3.39	.042						149	97	65.0	5.04	.052	15.6	2.32
1.03							18	14		1.99			
23.3%							10.8%	12.6%		28.3%			

chloride loss produced by fasting and water deprivation is 13.8 per cent of the initial body content, and roughly one-third of this, and also of the water loss, is derived from the skin. The per cent losses from the skin are, it may be noted, about twice as great as those from the tissues.

When, added to the circumstances of fasting and water deprivation, a loss of gastric secretions is produced by placing an obstruction at the pylorus, body weight falls in 12 hours from 192 gm. to 181 gm. (average for three animals). But insomuch as the final weight (181 gm.)

includes the gastric secretions trapped in the stomach, the 11 gm. of body weight loss here observed is presumably referable to the factors of fasting and water deprivation and agrees roughly with the loss of 13 gm. produced in the preceding experiment by these circumstances, in the absence of obstruction. On opening the abdomen the stomach was found enormously distended and to contain water to the surprising extent of 15.6 cc. (average of findings). If 15.6 gm. be added to the observed body weight loss, 11 gm., a total weight loss of 26.6 gm. is obtained. The estimated weight loss from skin, tissues, and blood taken together given in the table is 18 gm. The considerable discrepancy between these two data is, we believe, explained by absorption of water from the gastro-intestinal tract below the obstruction. Unfortunately weight measurements of the tract were not made but it was observed *post mortem* that, in striking contrast to the enormous stomach, the remainder of the gastro-intestinal tract was completely empty and shrunken to a tiny cord. The inference thus produced is that about 8 or 9 cc. of the water lost into the stomach is replaced by water absorbed from the intestines. This source of body fluid replenishment is, of course, soon exhausted but is here seen to be of an extent sufficient to cover about one-half of the water loss into the stomach during the first 12 hours following obstruction. Although the effect of the loss of gastric secretions on the water content of the body is thus greatly reduced, the estimations of net loss of weight and of water, 10.8 per cent and 12.6 per cent respectively, indicate an appreciably more extensive loss of water than of substances, and this is found to be true in terms of per cent water content for both skin and tissues. The chloride loss is, as would be expected, greatly increased as compared with the loss found for fasting and water deprivation without obstruction, being nearly one-third of the initial chloride content of the body, and is completely accounted for by the amount found in the stomach. An extensive fall of chloride ion concentration is found in the skin and a considerable decrease occurs also in the blood, changes obviously referable to the much higher concentration of chloride ion in the gastric secretions than in body fluids.

These data enable us to appraise the two factors of water removal from the body in the presence of upper intestinal obstruction; (1) water leaving the body as a "waste product" of the consumption of

protoplasm due to the circumstance of fasting and, (2) water lost into the stomach because of the failure of reabsorption of gastric secretions. According to the results of these experiments the first factor is of the same magnitude as would be produced by fasting and water deprivation in the absence of obstruction and when obstruction is present it accounts for more than half of the water loss over the experimental period here studied. The large size of this factor was to us a surprising finding. The data for skin and for tissues given in Table 1 are graphically presented in Fig. 1, Sections A and B, with the purpose of bringing out this point more clearly. It can here be appreciated that although loss of gastric secretions considerably accelerates the decline of the values measured, fasting with water deprivation is the larger factor in the situation.

As regards the loss of water due to the second factor, that is, to failure of reabsorption of the gastric secretions, there remains for consideration the interesting question of its source. It has been argued that this water derives entirely from interstitial body fluids (6). The data here given are not definitely informative on this point. Since the skin, because of its loosely areolar understructure, serves as a chief depot of interstitial fluid, a relatively much larger loss of water from the skin than from other body tissues would be expected. This was the case in these experiments. It may be computed from the data in Table 1 that three-fourths of the water loss referable to obstruction derives from the skin. The small remainder comes from tissues which, although they contain relatively less interstitial water, are in bulk more than twice that of the skin. It would therefore seem possible that the loss of water from the tissues referable to obstruction, which, as may be seen in the table, causes a just measurable reduction of per cent water content, is entirely composed of interstitial water. Unpublished data obtained by one of us (McKhann) bearing on this point may appropriately be inserted here. They consist in measurements of per cent water content of two parenchymatous tissues, heart muscle and brain, taken from rats dehydrated by repeated intraperitoneal injections of hypertonic (4.5 per cent) sodium chloride solution and are presented in Table 2. They show, especially in the case of brain tissue, a remarkably successful defense of the normal water content.

Data from another experiment measuring the changes found over a

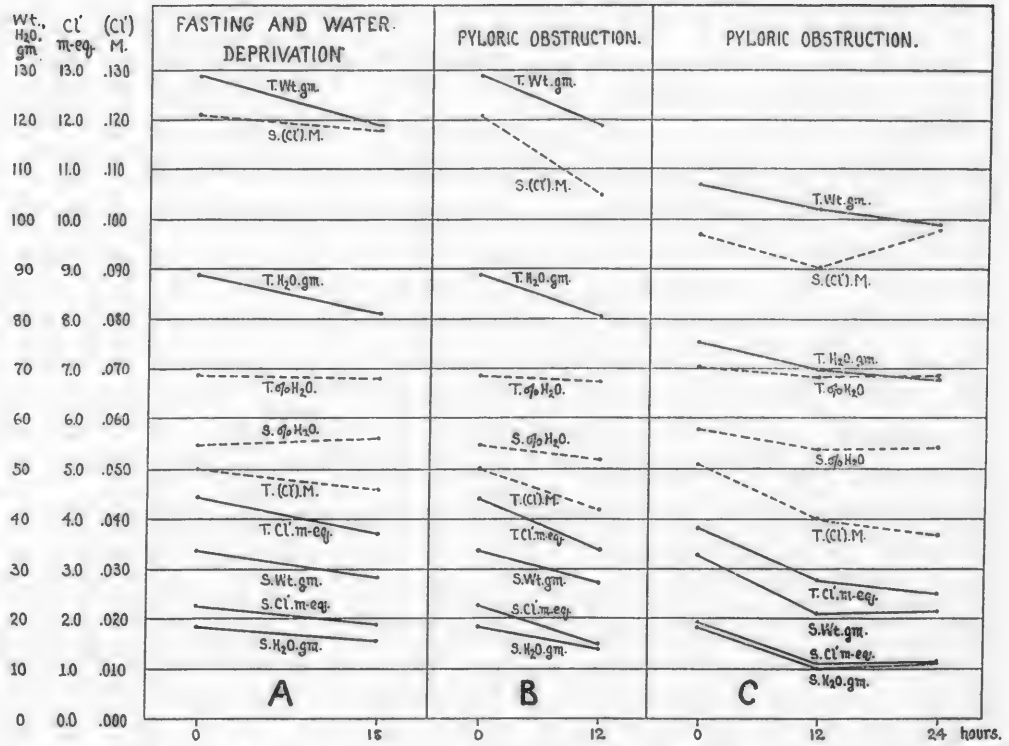

FIG. 1. Sections A and B present graphically the measurements of body weight, water, and chloride ion for skin (S) and for tissues (T) given in Table 1. The data plotted in Section C are from another series of animals and represent the effects of pyloric obstruction over a longer period (24 hours).

longer interval following pyloric obstruction are recorded in Section C of Fig. 1. These are from three rats of uniform weight (155 gm.). Striking decline in the rate of loss of water and chloride ion during the second 12 hours following obstruction is evident. Indeed, in the case of the skin, there is, according to the data, no further depletion of water and materials after the first 12 hours. Apparently by then the subcutaneous depots are completely exhausted and further losses must be entirely sustained by the other tissues. An extremely slight depression of the per cent water content of the tissues is again found.

In the first section of Fig. 2 are plotted measurements descriptive of the effects of prolonged fasting and water deprivation. After the

TABLE 2

Per Cent Water Content of Kidneys and Brains from Rats Dehydrated by Repeated Intra-Peritoneal Injections of Hypertonic Sodium Chloride Solution

Body weight, gms.		Water content, per cent	
Initial	Final	Kidneys	Brain
350	280	76.2	78.8
347	298	75.5	77.1
298	242	78.8	78.5
342	258	75.2	78.6
263	185	76.8	78.8
Averages of control data.....		77.2	78.5

initial 18 hour period a much less rapid rate of loss of body weight, water and chloride ion is established. The entire survival period was 187 hours. The total losses in per cent of initial values were as follows: body weight 39.6 per cent, water 38.3 per cent, chloride ion 35.3 per cent. A curious finding shown in the figure is the extensive rise of chloride ion concentration in the skin and the same change, though of much less degree, in the tissues. The per cent water content of the tissues remains nearly stationary, exhibiting a slight trend toward increment rather than depletion. In the skin an increase in per cent water content occurs more appreciably. In the experiment which produced the data given in the second section of Fig. 2, in addition to fasting and water deprivation, an obstruction

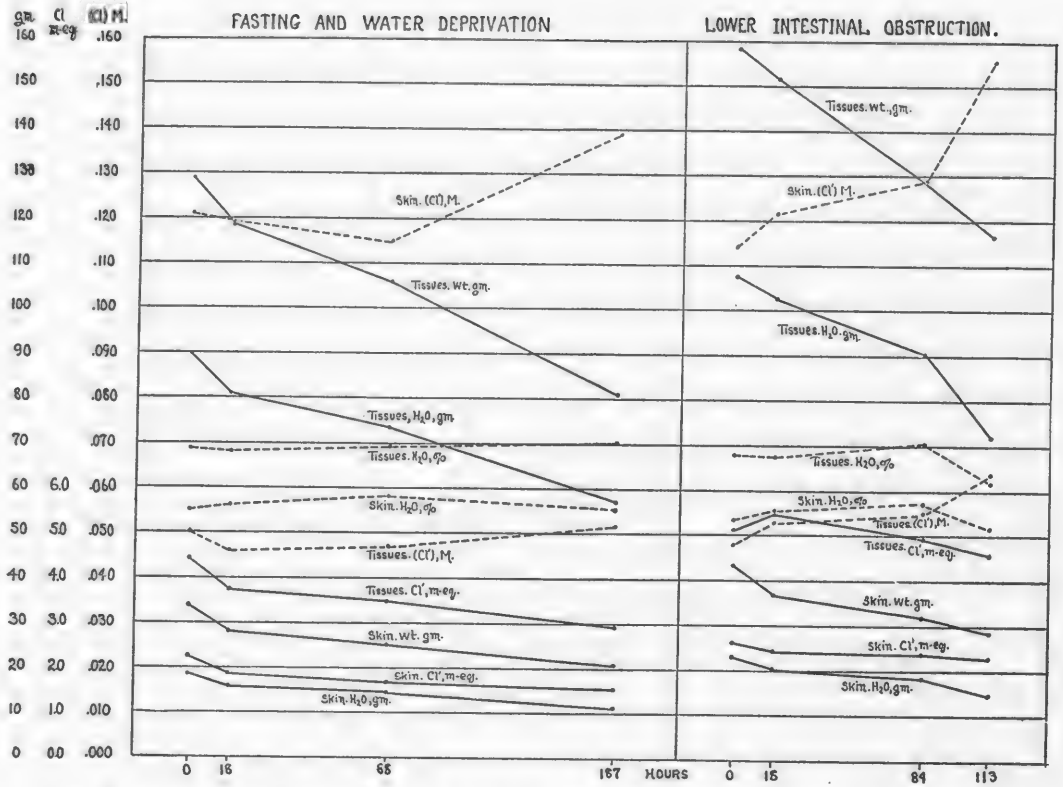

FIG. 2. Data representing effects of fasting with water deprivation, and of lower intestinal obstruction

was placed at the lower end of the small intestine. Gastro-intestinal secretions can presumably be reabsorbed. Evidence that they are is provided by the rise in the concentration of chloride ion even during the initial period where, as may be seen in Fig. 1, pyloric obstruction produces a sharp fall. The survival period as indicated by a single animal was shorter than for fasting with water deprivation alone, 113 hours as compared with 187 hours. The two sets of data are, however, similar to a degree which permits the inference that lower intestinal obstruction causes little if any water removal from the body.

SUMMARY

The circumstances present in upper intestinal obstruction which may be expected to reduce the water content of the body are fasting with water deprivation and a continued loss of secretions into the stomach. According to the data obtained from the above described experiments with rats, loss of body water during the first third of the survival period following pyloric obstruction is more than half accounted for by fasting with water deprivation. This body water is accompanied by a parallel loss of solids and may be regarded as a waste product of the consumption of body fat, glycogen, and protoplasm. Its loss does not disturb the per cent water content of the body tissues. The water lost into the stomach is responsible for an actual excess of water reduction over consumption of solids. Except in the case of the skin and blood, this excess loss of water is extremely small and produces a reduction of the per cent water content of tissues which is so slight as to permit the surmise that the water loss here derives entirely from the interstitial fluid of the tissues and that no dehydration of tissue cells occurs. The data are, however, not directly informative on this point. The total loss of body water during 12 hours following pyloric obstruction was found to be 12.6 per cent of the water content of a control animal.

More than one-quarter (28.3 per cent) of the total body content of chloride ion was found to be lost and was entirely accounted for by the amount of chloride found in the gastric contents. Nearly half of the chloride loss derives from the skin.

Data are presented which demonstrate that lower intestinal obstruction causes slight, if any, depletion of the water content of the body.

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STUDIES ON INFLAMMATION

III. Fixation of a Metal in Inflamed Areas*

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In a previous communication (1) it was demonstrated that trypan blue injected intravenously rapidly enters an inflamed area and is fixed there so that the dye fails to reach the regional lymph nodes. Subsequent studies (2) revealed that the rate of fall of concentration of the dye in the capillaries as it diffuses into the extra-capillary spaces is more rapid in an inflamed than in a normal area. This was interpreted as being the result of increased capillary permeability with inflammation.

The earlier literature on the subject has been reviewed elsewhere (1). With the demonstration that the dissemination of bacteria was retarded by an inflammatory reaction (3, 4, 5) and with the subsequent studies on the fixation of dyes in inflamed areas, it became of interest to observe the behavior of other substances both when injected directly into an inflamed area and when injected into the blood stream. In some unpublished results it was found that carbon particles in the form of India ink failed to reach the tributary lymph nodes when injected directly either into a subcutaneous area of inflammation or when injected into an inflamed peritoneal cavity. When, however, the ink was injected intravenously no definite evidence of carbon deposit in the inflamed area could be obtained.

At the suggestion of Dr. Eugene L. Opie studies were then undertaken to determine whether a metal injected intravenously would accumulate rapidly in an inflamed area and be fixed there in a manner similar to trypan blue.

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The Fixation of Iron by an Inflammatory Reaction in the Peritoneal Cavity

The observations were made with the use of iron, largely because this metal could readily be detected when in sufficient amount in the tissues by placing them in an acidified solution of potassium ferrocyanide (Prussian blue reaction).

TABLE I

Retention of Iron in Inflamed Peritoneal Cavity

Experiment	Interval between injection of irritant and that of iron*	Total duration of inflammation**	Prussian blue reaction in the retrosternal lymph nodes after injection of iron into the inflamed peritoneal cavity	Prussian blue reaction in the retrosternal lymph nodes after injection of iron into the normal peritoneal cavity
	hrs. : mins.	*hrs. : mins.*		
1	0 : 00	5 : 30	+	+
2	1 : 20	4 : 45	++	+++
3	3 : 05	5 : 45	0	+++
4	18 : 00	23 : 40	trace	++
5	21 : 30	28 : 00	0	+++
6	22 : 20	28 : 00	0	++
7	22 : 20	29 : 00	faint trace	+++
8	22 : 40	23 : 40	0	+++
9	23 : 15	28 : 00	0	++
10	23 : 15	28 : 45	faint trace	+
11	23 : 45	29 : 30	0	++
12	23 : 50	29 : 00	0	+
13	24 : 30	47 : 30	0	+++

0 indicates no Prussian blue reaction; + a pale blue reaction; ++ a conspicuous blue reaction; +++ a deep blue reaction.

* In all except one experiment dialyzed iron was used. In Experiment 8 ferric chloride was used.

** In all except one experiment aleuronat was used as the inflammatory irritant. In Experiment 4 a suspension of *Staphylococcus aureus* was used as the inflammatory irritant.

Opie (5) recently showed that acute inflammation of the peritoneal cavity caused by aleuronat retards the rush of injected hemolytic streptococci from the peritoneal cavity into the circulating blood and after 24 hours completely prevents it. Subsequently it was found (1) that trypan blue injected into the normal peritoneal cavity rapidly

appears in the lymph of the retrosternal lymphatics and stains deeply the retrosternal lymphatic nodes. Trypan blue, on the other hand, injected into an inflamed peritoneal cavity is fixed *in situ* and fails to reach the retrosternal lymphatic nodes.

5 cc. of dialyzed iron* were injected into the peritoneal cavity of normal rabbits and also into that of rabbits with acute inflammation caused, with one exception, by a previous injection of 5 per cent aleuronat and 3 per cent starch in 0.5 per cent saline. The thorax was opened at various intervals after the injection of the metal and the retrosternal region dipped in 2 per cent potassium ferrocyanide and 1 per cent hydrochloric acid. Aleuronat *per se* gives a Prussian blue reaction but the evidence that none of the irritant appeared in the retrosternal lymphatic nodes is clear since in the animals with inflamed peritoneal cavity the retrosternal lymph nodes showed no Prussian blue reaction. The results are shown in Table I.

It is seen that when iron was injected immediately after the inflammatory irritant there was no retention of it (Experiment 1). When, however, the inflammatory reaction had been in progress for as long as 3 hours the metal was fixed and failed to reach the retrosternal lymphatic nodes. The injection of the metal into the normal peritoneal cavity was followed on the other hand by its appearance in the retrosternal lymphatic nodes. *Staphylococcus aureus* was used as the inflammatory irritant in one experiment (No. 4) with similar results, *i.e.*, fixation of the metal *in situ*. Ferric chloride (No. 8) behaved exactly as did dialyzed iron. In the inflamed peritoneal cavity where retention of the metal took place, large clumps and masses of brown deposits were often seen on the omentum and on the peritoneal surface of the diaphragm.

Having established qualitatively that colloidal iron and, in one instance, that ferric chloride are retained *in situ* by the inflammatory reaction, it became of interest to determine quantitatively the amount of fixation of the metal.

Quantitative determinations of iron in tissue were performed according to the method of Kennedy (6) with only slight modifications. 5 cc. of ferric chloride (0.25 per cent solution in saline) were injected into the peritoneal cavity of a normal rabbit as control and also into the inflamed cavity of an experimental animal. 1 to 2½ hours later the animal was killed and the retrosternal lymph

* This product is colloidal ferric hydroxide with 5 per cent ferric oxide and ferric chloride.

nodes carefully removed, weighed, and placed in a 100 cc. Kjeldahl flask. 2 to 3 cc. of concentrated sulfuric acid and about 1 cc. of 60 per cent solution of perchloric acid were added. The mixture was heated over a low flame for about 10 minutes until complete digestion took place and the solution appeared almost colorless. While the mixture was still hot, 0.5 cc. of 30 per cent hydrogen peroxide was added and the mixture was cooled and diluted to 50 cc. 10 cc. of this solution were pipetted into a 50 cc. stoppered cylinder to which were added 10 cc. of amylic alcohol and 5 cc. of 20 per cent sodium thiocyanate. The mixture was immediately shaken and the iron was extracted by the amylic alcohol layer. This layer was pipetted off into a colorimeter cup and compared with a standard treated in exactly the same manner.

TABLE II

Amount of Iron in Retrosternal Lymph Nodes after Its Injection into Inflamed and Normal Peritoneal Cavity

Experiment	Interval between injection of irritant and that of iron	Total duration of inflammation	Amount* of iron in nodes after its injection into inflamed peritoneal cavity	Amount of iron in nodes after its injection into normal peritoneal cavity	Per cent of difference in iron content
	hrs. : mins.	*hrs. : mins.*			
1	17 : 30	18 : 30	165.7	222.7	25.6
2	22 : 30	25 : 00	195.7	762.5	74.3
3	22 : 30	25 : 00	38.5	180.95	78.7
4	24 : 30	26 : 00	86.0	127.0	48.0
Average of per cent difference in iron content.....					56.7

* Figures are expressed in milligrams of iron per 100 gm. of tissue.

The results in Table II show that fixation of iron by an inflammatory process varies a good deal in different animals. These variations may partly be due to the fact that the determinations of iron content were performed by the "wet ashing" method, and probable differences in the amount of fluid in lymph nodes draining an inflamed and normal peritoneal cavity were therefore not taken into consideration in the final determinations. The average amount of iron in the retrosternal lymphatic nodes of normal animals is 56.7 per cent higher than in the nodes of animals with inflamed peritoneal cavity.

Three determinations were also performed to determine the iron content of retrosternal lymph nodes in animals receiving no iron. Two normal animals showed 35.6 and 44.8 mg. of iron per 100 gm. of

TABLE III

Accumulation of Iron at the Site of Cutaneous Inflammation

Experiment	Duration of inflammation	Prussian blue reaction in inflamed skin areas of animals that received ferric chloride	Prussian blue reaction in inflamed skin areas of animals that receive no iron
	hrs. : mins.		
1	1 : 45	trace	0
		+	
		+	
		faint trace	
2	4 : 30	++	0
		++	0
		+	0
		+	0
3	6 : 15	+++	0
		++	0
4	6 : 30	++	0
		+	0
5	8 : 00	trace to +	0
6	11 : 00	++	0
		++	0
		++	0
		++	
7	23 : 30	+	0
		++	0
		++	faint trace
		+	
8	48 : 00		0
			0
9	70 : 00		++
			++
10	168 : 00		+++
			+++

0 indicates no Prussian blue reaction; + a pale blue reaction; ++ a conspicuous blue reaction; +++ a deep blue reaction.

tissue. One determination was made on the lymph nodes of an animal that had received no iron but had an inflamed peritoneal cavity. In this animal the value for iron content was 68.6 mg. per 100 gm. of tissue. These results with non-injected animals show that lymph nodes normally contain an appreciable quantity of iron, which is evidently bound up in a form that does not give the Prussian blue reaction. The Prussian blue reaction is less sensitive than the thiocyanate test for the detection of iron. Furthermore hemoglobin does not respond to the ordinary chemical test for iron. This probably accounts for the iron content of normal lymph nodes giving a negative Prussian blue reaction.

The above results show that like trypan blue, iron is fixed *in situ* by the inflamed cavity and much less of it reaches the retrosternal lymphatic nodes than under ordinary circumstances.

The Accumulation of Iron Injected into the Circulating Blood in Inflamed Areas

It having been found that iron was fixed *in situ* when injected directly into an inflamed area, studies were undertaken to determine whether, as in the case of trypan blue, the metal would accumulate in an inflamed area when introduced into the circulating blood stream.

25 mg. of ferric chloride in saline were injected intravenously. The areas of inflammation were caused by several injections of 0.1 to 0.2 cc. of a saline suspension of *Staphylococcus aureus* into the dermis of the abdomen of rabbits. The accumulation of iron was studied after the inflammatory reaction had been under way for various lengths of time. Inasmuch as it was found that an inflamed area *per se* of 70 hours duration or more gives a positive Prussian blue reaction owing doubtless to pigment from red blood corpuscles escaped by diapedesis or capillary hemorrhage, studies of iron accumulation in inflammation when ferric chloride was injected intravenously were not carried beyond 24 hours. The results are shown in Table III.

It is clear that the metallic salt accumulates in an inflamed area when the inflammatory process has lasted only 1 hour and 45 minutes. On the other hand the inflamed areas in non-injected animals give no qualitative test for iron within the first 11 hours of inflammation at least. The difference in the two sets of animals is conspicuous.

It is interesting to note that an old inflammatory lesion in non-

injected animals of at least 70 hours duration shows the presence of iron by the qualitative test. This is probably due to the degradation in the late stages of inflammation of hemoglobin from red corpuscles phagocytosed by tissue macrophages. Histological sections of such areas reveal within large mononuclear cells hemosiderin which gives the Prussian blue reaction. A similar observation in animals was recently made by Polson (7) while studying the fate of colloidal iron administered intravenously. However, this author does not refer it to the administration of iron. He was dealing with an inflammatory

TABLE IV

Amount of Iron in Inflamed Areas of Animals That Received No Iron and of Animals Injected with Ferric Chloride*

Experiment	Injected animals		Non-injected animals	
	Normal skin areas	Inflamed skin areas	Normal skin areas	Inflamed skin areas
1	9.3	13.0	8.4	9.2
2	12.5	15.7	8.4	9.4
3	7.7	15.5	5.6	9.6
4	16.6	29.6	10.9	9.0
5	6.9	10.6	7.9	11.2
6	9.7	12.7	9.2	9.9
Average.....	10.4	16.2	8.4	9.7

* Figures are expressed in milligrams of iron per 100 gm. of dry tissue.

focus of relatively long duration which showed a large amount of iron in spite of the fact that no metal was injected.

Quantitative studies were undertaken to determine the exact amount of accumulation of metal in inflamed areas when 50 mg. of ferric chloride (Fe_2Cl_6, 12 H_2O) in distilled water were injected intravenously.

The inflamed areas were obtained by the injection into the dermis of the abdomen of about 0.2 cc. of a suspension of *Staphylococcus aureus* in broth concentrated to one-tenth of its original volume. 6 to 7 hours later the animal was killed and the inflamed areas were carefully removed, the subcutaneous tissue adhering to them being discarded. Control areas of normal skin in the same animal were also removed from the abdomen. These areas were then placed in an electric dry oven for about 16 hours at 100°C. When thoroughly dried the tissues were

weighed and digested by the method described above with the exception that now larger quantities of sulfuric and perchloric acids were used, the values being 8 and 5 cc. respectively. By determining the iron content of tissues on the dry basis, variations in the final results due to differences in fluid content of inflamed areas were largely eliminated. Thus a more accurate method of comparison between iron content of normal skin and inflamed areas was obtained. It is evident that with diapedesis of red corpuscles there is probably always more iron in an inflamed area than in a normal tissue area. For this reason an additional series of control animals was studied for iron content exactly as above with the exception that none of these rabbits received any intravenous injection of ferric chloride. The results obtained for iron content in inflamed areas in both groups of animals are shown in Table IV.

From this table it is seen that the average iron content in inflamed areas of animals injected with ferric chloride is 16.2 mg. per 100 gm. of dry tissue as compared with 9.7 mg. in inflamed areas of animals that received no metal. Consequently the amount of iron in inflamed areas is increased 67 per cent by injection of 50 mg. of ferric chloride in the blood stream. A comparison of the iron content of normal skin areas in injected and non-injected animals, 10.4 and 8.4 mg. respectively, shows that the accumulation in normal tissues resulting from the injection of the metal is only 23.8 per cent. Hence the accumulation of iron following intravenous injection of ferric chloride is about three times greater in inflamed than in normal skin areas. The iron recovered from inflamed areas of an animal not injected with this metal is contained in hemoglobin which does not give the Prussian blue reaction. This accounts for the negative reaction when the qualitative test is applied in these animals to inflamed areas of relatively short duration.

These experiments suggest that fixation of a metal or dye in an inflamed area may have clinical application. It is conceivable that dyes, iron containing substances, and various other materials by their accumulation at the site of inflammation may alter the character or course of the inflammatory reaction.

CONCLUSIONS

Colloidal iron or ferric chloride injected into the inflamed peritoneal cavity is fixed in the cavity and fails to reach the retrosternal lymphatic nodes, whereas, in the absence of inflammation, iron accumulates

in these nodes and becomes demonstrable by the Prussian blue reaction.

Quantitative studies show that after intraperitoneal injection of ferric chloride the retrosternal lymphatic nodes of animals with normal peritoneal cavity contain approximately 56 per cent more iron than do the nodes of animals with inflamed peritoneal cavity.

Ferric chloride injected into the circulating blood enters an inflamed area in the skin and the inflamed tissue gives the Prussian blue reaction. Quantitative determinations show that the amount of iron in inflamed areas is much greater than that found in inflamed areas of animals that have received no iron.

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ACCUMULATION OF ANTIBODIES IN THE CENTRAL NERVOUS SYSTEM

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The purpose of the present paper is to describe the accumulation of antibodies in the central nervous system of rabbits. Information on this subject may throw light upon several important problems: (1) the penetration of protein (globulin) from the blood into the spinal fluid, brain and cord, for antibodies cannot be separated from globulins experimentally, (2) the relationship between the cerebrospinal fluid and the tissue fluid of the brain and spinal cord and (3) the prevention and treatment of certain diseases of the central nervous system by the injection of immune serums.

The present work is a continuation of a series of studies (1) on the antibody content of the serum and organs of rabbits, which have yielded information as to the distribution of agglutinins in the serum, organs and lymph. Agglutinins against typhoid bacilli were selected as antibodies because they can be obtained in high concentration and can be measured easily and with relative accuracy. The rabbit was employed because the titers of the immune serums of rabbits are very much higher than in other small laboratory animals.* The experiments were carried out on actively and passively immunized animals. The active immunization consisted of a series of injections of killed typhoid bacilli into an ear vein. The passively immunized rabbits received one injection of immune serum into the blood stream. The antibody content of the organs was measured by extracting the organs (after grinding with sand) with salt solution and titrating the extracts. For the titration of agglutinins a method was used that is more sensitive than the usual routine method of agglutination. This method consists in centrifugalizing the tubes containing the serum or extract-dilutions and bacteria and reading the results while the tubes are being gently shaken and the sedimented bacteria resus-

* The dog—an animal extensively used in investigations on the central nervous system—does not furnish potent serum. Dogs could have been injected in the passive immunization experiments but by injecting immune serum obtained from rabbits into other rabbits the introduction of foreign protein is avoided.

pended. This technic was first used by Gathgens (2) in 1906 and has been employed by a number of investigators (3) since that time. For further technical details see previous publications of the present series.

The main results of the work, as already reported, can be summarized as follows: When serum containing antibodies is injected into an ear vein of the normal rabbit, antibodies accumulate in all the organs studied: the liver, spleen, kidney, lung, skin, muscle and uterus (smooth muscle). The rate of accumulation varies in the different organs. The final concentration is reached in the liver, spleen, lung and kidney in less than 10 minutes after the injection of immune serum, but in the uterus and skin only after several hours have elapsed. The antibodies penetrate most slowly into the skin. When the final amount of antibodies is accumulated there is a constant numerical relationship between the antibody content of the blood and organs. On the average, the highest dilution of extract prepared from 1 gm. of liver, spleen, kidney, lung or skin that agglutinated typhoid bacilli was one-tenth of the highest dilution of 1 cc. of serum that agglutinated typhoid bacilli.

Since there are less antibodies in the organs than in similar amounts of blood the question naturally arises whether the antibodies recovered from the organs are due to the blood present in them. That they are not derived mainly from the blood of the organs but from the extravascular part of the tissue is evidenced by the following observations. (1) Lymph obtained by cannulating the lymph ducts of the liver, leg, neck and thoracic duct contains antibodies in higher concentration than the organ extracts. (2) Perfusion does not reduce the antibody content of the skin and uterus. (3) More antibodies can be recovered in the perfusate from the living animal than were present in the blood before perfusion, showing that during perfusion antibodies penetrate from the organs into the blood vessels, an observation recently confirmed by Schwarzmann (4).

The equilibrium between the antibody content of the blood and of the organs can be reached from either the blood or the organs, for an identical relationship will establish itself between the antibody content of the serum and organs when the immune serum is injected either into the blood stream or into the skin.

EXPERIMENTAL

The experiments to be reported here were performed with both actively and passively immunized rabbits for these reasons. Passive immunization offers an opportunity to establish the rate of accumulation of antibodies in the organs and cerebrospinal fluid by examination of the rabbits at different intervals of time after the injection of immune serum. In actively immunized rabbits the titers of the blood and organ extracts are higher and therefore the observations are more striking. The technic of immunization was the same as in the previous work. The immune serum used was fresh, and was obtained and kept under sterile conditions.

Before describing the experiments, it must be emphasized again that the agglutination tests were made with the aid of centrifugalization, a method that is more sensitive than the routine method of agglutination. Without this technic, the results described in the present study cannot be duplicated.

I. Antibody-Content of the Brain, Spinal Cord and Cerebrospinal Fluid (from Cisterna Magna) of Actively Immunized Rabbits

The technic of these experiments differed from that of the earlier experiments in that urethane was not used for anesthesia because it is said that it promotes the

TABLE I

Agglutinin Titers of the Serum, Spinal Fluid, Brain and Spinal Cord of Actively Immunized Rabbits

Number of rabbit	Serum	Spinal fluid	Brain	Cord
1	1:150,000	1:640	1:2,500	1:1,300
2	1:150,000	1:420	1:600	1:384
3	1:128,000	1:160	—	—
4	1:102,000	1:512	1:576	1:1,200
5	1:102,000	1:256	1:1,500	1:362
6	1:102,000	1:512	—	—
7	1:102,000	1:512	—	—
8	1:102,000	1:512	1:583	1:290
9	1:50,000	1:128	—	—
10	1:32,000	1:80	1:200	1:104
11	1:32,000	—	1:192	1:60
12	1:32,000	1:40	—	—
13	1:26,000	1:48	—	—
14	1:20,000	1:52	—	—
15	1:13,000	1:26	—	—
16	1:3,200	1:5	—	—

passage of substances into the spinal fluid. The animals were narcotized with the minimum amount of ether necessary. Some of the rabbits were bled to death from the left carotid artery; some from the femoral arteries and the descending aorta; the site of bleeding did not influence the results. After the rabbits were bled to death cerebrospinal fluid was removed from the cisterna magna by means of a tuberculin syringe and skin-test needle (gauge 22). After some practice there was no difficulty in obtaining fluid free of blood. The samples of cerebrospinal fluid—in most cases 0.4 cc.—were centrifugalized and the sediment examined under a

microscope. Samples containing more than one red blood cell in 10 microscopic fields (seen with high dry lens) were rejected. From the brain and spinal cord the meninges were removed very carefully and the ventricles of the brain and central canal of the cord were then opened and carefully rinsed with salt solution. To ascertain whether any cerebrospinal or other fluid containing agglutinins was left on the surface of the organs, the last washing fluid was examined for agglutinins. Some of the washing fluid caused a trace of agglutination, but dilutions 1 in 2, 1

TABLE II

Agglutinin Titers of the Spinal Fluid and of Extracts of the Brain and Spinal Cord of Actively Immunized Rabbits*

Number of rabbit	Spinal fluid	Brain extract	Spinal cord extract
	per cent	*per cent*	*per cent*
1	0.43	1.70	0.87
2	0.28	0.40	0.26
3	0.12	—	—
4	0.50	0.56	1.17
5	0.25	1.47	0.35
6	0.50	—	—
7	0.50	—	—
8	0.50	0.57	0.28
9	0.26	—	—
10	0.25	0.62	0.32
11	—	0.60	0.18
12	0.12	—	—
13	0.18	—	—
14	0.26	—	—
15	0.20	—	—
16	0.20	—	—
Average.....	0.33	0.82	0.49

* The titers are expressed as percentages of the titers of the serum.

in 4 did not agglutinate typhoid bacilli. The washed organs were dried by pressing them lightly between filter papers, weighed, and ground with sea sand alone and with saline. The extracts were centrifugalized at high speed, the sediment discarded, and the supernatant fluid centrifugalized again until it became clear. In recording the results of titration, titer 1:100 means that when 1 gm. of brain was extracted with 9 cc. saline, the extract, diluted ten times, clumped typhoid bacilli and a dilution twice higher—1:200—did not agglutinate typhoid bacilli.

Tables I and II show the following.

1. The cerebrospinal fluid of all the rabbits immunized with killed

typhoid bacteria contained antibodies. The titer of the cerebrospinal fluid varied with the titer of the serum, the ratio of the titer of the serum to the titer of the cerebrospinal fluid being, on an average, 300:1, or 0.33 per cent. The variation in this numerical relationship exceeded in one case only the limit of accuracy of the method. One should bear in mind, in this connection, that in the agglutination test the serum, cerebrospinal fluid and organ extract are diluted by halves (1 in 100, 1:200).

2. The brain and the spinal cord of all the actively immunized animals contained agglutinins. The titer of the extracts of the brain and cord varied with the titer of the serums, but the numerical relationship between the titers of the serums and organ extracts was not so consistent as the numerical relationship between the titers of the serums and the cerebrospinal fluid. The ratio of the titers of the serums to the titers of the extracts of the brain and of the spinal cord ranged from 100:0.4 to 100:1.7 and from 100:0.18 to 100:1.17 respectively. The average agglutinin titer of brain extract was 0.82 per cent (of the titer of the serum) and that of the spinal-cord extract 0.49 per cent (of the titer of the serum). Therefore extracts prepared from 1 gm. of brain or spinal cord were more potent than the dilution of a similar amount of cerebrospinal fluid.

Summarizing the results of the observations on actively immunized rabbits, the following average numerical relationship was found between the agglutinin titers of the serum, spinal fluid, brain and spinal-cord extracts:

Blood serum	100. per cent
Cerebrospinal fluid	0.33 per cent
Brain extract	0.82 per cent
Spinal-cord extract	0.49 per cent

II. Antibody-Content of the Central Nervous System of Passively Immunized Rabbits

In studies of the distribution of antibodies in the blood and organs of passively immunized animals it is of great advantage to employ immune serums of high titers; therefore only very potent serums were used for passive immunization. The majority of the animals were injected with immune serums of which the titer was 1:64,000. The titer of one of the immune serums was as high as 1:200,000.

The titer of the serums and the amount injected are given in Table III. The serum was introduced slowly into an ear vein through a skin-test needle (gauge 22), the injection lasting from 3 to 4 minutes. Since it is generally believed that substances in solution injected into a peripheral vein are evenly distributed in the blood stream within 3 minutes, six rabbits were bled to death as early as 15 minutes after the immune serum had been injected. The results of the titrations of the serums, cerebrospinal fluids and organ extracts are tabulated in Tables III and IV.

TABLE III

Agglutinin Titers of the Serum, Spinal Fluid, Extracts of Brain and Spinal Cord of Passively Immunized Rabbits

Number of rabbit	Serum injected		Time between injection of serum and examination	Serum	Spinal fluid	Extract of brain	Extract of spina cord
	Amount	Titer					
1	10 cc.	1:128,000	15 minutes	1:25,000	0	1:80	1:80
2	10 cc.	1:128,000	15 minutes	1:25,000	0	1:50	1:50
3	25 cc.	1:64,000	15 minutes	1:16,000	1:5	1:96	1:29
4	25 cc.	1:64,000	15 minutes	1:24,000	less than 1:2.5	1:240	1:84
5	15 cc.	1:64,000	15 minutes	1:12,000	1:6	1:264	1:180
6	15 cc.	1:64,000	15 minute	1:12,000	less than 1:12	—	—
7	10 cc.	1:128,000	2 hours	1:18,000	1:1.8	1:68	1:18
8	10 cc.	1:128,000	2 hours	1:25,000	—	1:120	1:50
9	10 cc.	1:80,000	2 hours	1:10,000	1:6	1:120	1:80
10	25 cc.	1:64,000	3 hours	1:12,000	1:3.6	—	—
11	20 cc.	1:64,000	3 hours	1:12,000	1:32	—	—
12	15 cc.	1:200,000	4 hours	1:32,000	1:19	1:294	1:126
13	20 cc.	1:64,000	18 hours	1:6,400	1:11	1:29	1:10
14	25 cc.	1:64,000	18 hours	1:3,200	1:8	1:13	1:5
15	20 cc.	1:32,000	20 hours	1:3,200	1:3.2	—	—
16	20 cc.	1:64,000	20 hours	1:4,800	1:18	—	—
17	20 cc.	1:64,000	20 hours	1:3,200	1:10	—	—
18	20 cc.	1:64,000	24 hours	1:3,200	1:11.5	—	—
19	20 cc.	1:200,000	24 hours	1:40,000	1:160	—	—

The tables show that in four rabbits, which were bled beginning 10 to 15 minutes after the injection of immune serum and ending 10 minutes later, the undiluted cerebrospinal fluid failed to agglutinate typhoid bacilli. However, in two other rabbits a very small amount of agglutinins penetrated into the cerebrospinal fluid within that time. The penetration of the antibodies into the cerebrospinal fluid (obtained from the cisterna magna) proceeded at a slow rate, for the high-

est titers in the cerebrospinal fluid were found only after 15 hours had elapsed following the injection of immune serum. In these animals the titer of the cerebrospinal fluid ranged between 0.10 to 0.40 per cent of the titer of the serum; that is, it was about as high as in actively immunized rabbits.

TABLE IV

Agglutinin Titers of the Cerebrospinal Fluid and of Extracts of the Brain and Spinal Cord of Rabbits Following Intravenous Injection of Immune Serum*

Number of rabbit	Time between injection of serum and examination	Titer of cerebrospinal fluid	Titer of brain extract	Titer of extracts of spinal cord
		per cent	*per cent*	*per cent*
1	15 minutes	0	0.32	0.32
2	15 minutes	0	0.20	0.20
3	15 minutes	0.03	0.60	0.18
4	15 minutes	less than 0.01	1.00	0.35
5	15 minutes	0.05	2.2	1.5
6	15 minutes	less than 0.1	—	—
7	2 hours	0.01	0.38	0.10
8	2 hours	—	0.48	0.20
9	2 hours	0.06	1.20	0.80
10	3 hours	0.03	—	—
11	3 hours	0.27	—	—
12	4 hours	0.06	0.92	0.43
13	18 hours	0.17	0.45	0.15
14	18 hours	0.25	0.40	0.14
15	20 hours	0.10	—	—
16	20 hours	0.37	—	—
17	20 hours	0.31	—	—
18	24 hours	0.36	—	—
19	24 hours	0.40	—	—

* The titers are expressed as percentages of the titers of the serums.

Tables III and IV show that just as in actively immunized rabbits, the extracts of the brain and cord of passively immunized rabbits contained agglutinins. The numerical relationship of the titers of the serums and extracts of brain and cord are very similar to those found in the actively immunized animals, the titer of brain extract being on an average 0.70 per cent and that of the cord 0.71 per cent of the titers of the serum. The variation from the average was more marked than in the actively immunized rabbits.

It was expected that the accumulation of antibodies in the central nervous system would proceed at a slow rate. This was found true of the penetration of agglutinins into the cerebrospinal fluid. However it had not been expected that the titers of the organ extracts of rabbits examined 15 minutes after the injection of immune serum would be as high as those from rabbits that had been in contact with immune serum for 24 hours. This unexpected observation suggests either that the penetration of antibodies into the brain and spinal cord (tissue fluid of these organs) is as fast as into some organs, such as the spleen, liver and lung and faster than into the uterus and skin, or that the antibodies recovered were due mainly to the blood present

TABLE V

Antibody Titers of Serums and of Extracts of the Perfused Brain of Actively Immunized Rabbits

Number of rabbit	Titer of serum	Titer of brain extract	Titer* of brain
			per cent
1	25,000	270	1.0
2	25,000	180	0.7
3	12,800	770	0.6
4	250,000	1280	0.5
5	128,000	640	0.5

* Expressed as percentages of the titers of the serums.

in the brain and spinal cord. To throw light on this question, I perfused the brain of rabbits of which the blood serum had a high titer in the agglutinin test.

III. Antibody-Content of the Cerebrospinal Fluid, Brain and Spinal Cord after Perfusion

The perfusion experiments were performed as follows. An actively immunized rabbit was lightly narcotized with ether, and about 30 cc. of blood was obtained from the left femoral artery. Then 0.15 gm. of heparin was injected into an ear vein; 3 minutes later the rabbit was bled to death from the femoral arteries and the abdominal part of the descending aorta. Immediately after the bleeding was completed, the brachial arteries and descending part of the aorta were ligated, and Locke solution at 42°C. was introduced into the arch of the aorta with the purpose of perfusing the brain through the vertebral and internal carotid arteries. The

perfusion was usually continued for about 1 hour; about 500 cc. of perfusion fluid was used. The examination of cerebrospinal fluid, and of organ extract was carried out as described above.

Table V shows that perfusion did not diminish the antibody titers of the brain extract or the cerebrospinal fluid. However it was felt that before drawing a conclusion from this observation as to the presence of the antibodies in the extra-vascular brain tissue, it was desirable to obtain evidence of the adequacy of the perfusion experiments. To this end I compared histological sections of the brains of the exsanguinated rabbits with those prepared from rabbits whose brain was perfused. The comparison showed that in the unperfused brains the majority of the blood vessels contained red blood cells whereas in the perfused brains the blood vessels were distended and red blood cells were absent from the majority of them.

Final evidence for the view that the antibodies recovered from the brain by extraction are derived from the extra-vascular part of the tissue would be the demonstration of antibodies in the lymph flowing from the brain. However, no lymph duct draining the brain is known; therefore no direct evidence can be furnished at the present time for this view.

DISCUSSION

The antibody content of the cerebrospinal fluid of normal and diseased human beings and lower animals has been the subject of extensive clinical and experimental investigations. It has been very generally accepted that antibodies do not penetrate from the blood into the cerebrospinal fluid unless the meninges are inflamed. A survey of the literature, however, shows that several authors have reported the presence of antibodies in the cerebrospinal fluid without inflammation.

Hektoen and Carlson (5) found opsonins but no haemagglutinins in the cerebrospinal fluid of actively or passively immunized dogs. Becht and Greer (6) could not demonstrate agglutinins in the cerebrospinal fluid of rabbits immunized with typhoid vaccine. Kafka (7) found traces of hemolysins and bacterial agglutinins in the cerebrospinal fluid of immunized dogs. Starkenstein and Zitterbart (8) reported that only undiluted cerebrospinal fluid of dogs agglutinated typhoid bacilli, although the titer of their serum was as high as 1:10,000.

There are two reports in the literature on the relative titers of tetanus antitoxin in the serum and cerebrospinal fluid. Ransom (9), working in von Behring's laboratory, compared the antitoxin titers of the serum and of the cerebrospinal fluid of one very highly immunized horse. He found that the ratio of the antitoxin titer of the serum of this animal to that of the cerebrospinal fluid was 100:0.4. Lemaire and Debre (10), who studied the effect of morphine upon the permeability of the meninges, reported that in dogs injected with tetanus antitoxin the ratio of the titer of the serum to that of the cerebrospinal fluid was 100:0.2.

The experiments performed in the present study show clearly that antibodies are present in the cerebrospinal fluid of actively or passively immunized animals without inflammation of the meninges. The possible objection that the antibodies demonstrated were due to inflammation or contamination of the cerebrospinal fluid with blood as a result of faulty technic can be met as follows. (1) The specimens of cerebrospinal fluid did not contain red blood cells at all or only in a negligible number. (2) There was a constant numerical relationship of the titers of serums to the titers of cerebrospinal fluids. (3) In passively immunized rabbits the antibody titer of the cerebrospinal fluid increased during the first 15 hours following the injection of immune serum, although the titer of the blood decreased during this time.

It is pertinent to inquire whether the central nervous system of the rabbits used in these experiments was free of pathological changes. McCartney (11) at the suggestion of Flexner, examined the brains of a large number of apparently healthy rabbits and found histological evidence of meningo-encephalitic lesions in more than 50 per cent. His observation is in conformity with those made in other laboratories (Bull (12), Oliver (13)).

The brains of rabbits employed in the present work were not examined histologically, but it is reasonable to assume that the lesions found by McCartney occur in the brains of our stock rabbits.

McCartney, Bull and Oliver did not study the spinal fluid of their rabbits and therefore it is not known whether the lesions found by them in the brain are associated with a large number of leucocytes or with other signs of inflammation in the cerebrospinal fluid. Although the spinal fluid of the rabbits employed in the present work was free from an abnormal number of leucocytes, the possibility cannot be excluded that meningo-encephalitic lesions so prevalent in apparently normal

rabbits did not influence the accumulation of antibodies in the central nervous system in some of them. However antibodies were found in the spinal fluid, brain and spinal cord in all of the actively and all of the passively immunized rabbits 2 hours after passive immunization. It hardly seems possible that the accumulation of antibodies in the central nervous system of all the rabbits could have been due solely to the presence of meningo-encephalitis.

The reasons for the general belief that antibodies do not penetrate from the blood into the cerebrospinal fluid without the inflammation of the meninges are probably the following. (1) The antibody titer of the cerebrospinal fluid is relatively low, as would naturally follow from the circumstance that the antibody titers of the serums of animals examined in the course of the reported studies were not high. (2) A large number of studies dealt with normal hemolysins, whose titer in the serum is very low.

Amoss and Eberson (14) reported that "agglutinins were not found in the spinal fluid of normal monkeys which had received antimeningococcic serum intravenously." This observation can be readily explained by the experiments on rabbits reported here, which show a ratio of 300:1 between the titers of the serum and of the spinal fluid; whereas the serum of the monkey in the experiment of Amoss and Eberson contained only 100 units of agglutinins per cubic centimeter, and the amount of agglutinins present in the spinal fluid was, therefore, too small to be detected. Flexner, Clark and Amoss (15) found that "it is unusual for the neutralizing principles to be contained in the cerebrospinal fluid during convalescence from epidemic poliomyelitis," although neutralizing principles are present in the blood. It is, however, not probable that the serums of the convalescents contained the neutralizing principles or antibodies in quantities that could be demonstrated in three-hundred-fold dilution.

Although, as my experiments show, antibodies penetrate from the blood into the spinal fluid of rabbits even without inflammation, the fact remains that the antibody content of the spinal fluid is very small. The results of numerous clinical and experimental observations have shown the effectiveness of sterile inflammation and of injecting immune serum into the spinal fluid in raising the antibody content of the spinal fluid (Flexner). In experiments to be reported later this finding has been reobtained in rabbits.

Since antibodies are found in the globulin fraction of the serum, behave in many respects like globulins and may be expected to follow the distribution of globulins, it is interesting to compare the ratio of the antibody titers of the serum and the cerebrospinal fluid on

the one hand with the ratio of the globulin of the serum and that of the cerebrospinal fluid on the other hand. Mestezrat (16) stated that the globulin content of the cerebrospinal fluid is about 0.019 gm. per 100 cc., and it is said that 100 cc. of serum contains on an average 2.5 gm. of globulin. These data were obtained from the serum and cerebrospinal fluid of man, but it is possible that the globulin content of the serum and cerebrospinal fluid in the rabbit is at least of similar magnitude if not almost equal to that found in human beings. The ratio of globulin content of the serum to that of the cerebrospinal fluid,

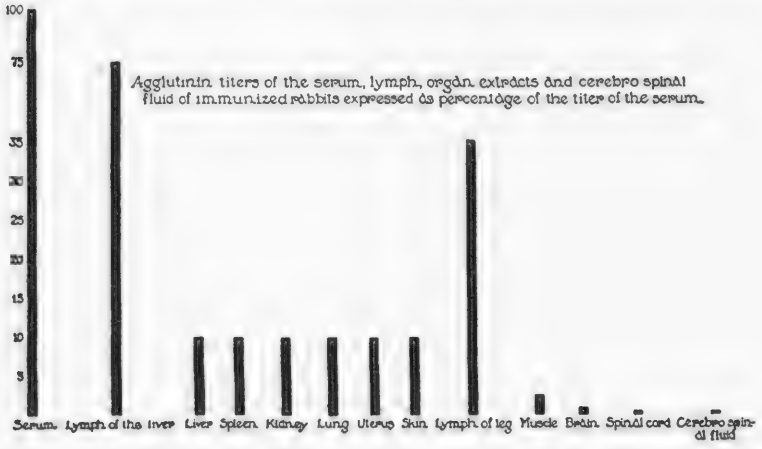

CHART 1

100:0.76, nearly equals that of the respective antibody titers, 100: 0.30. Considering the accuracy of the technic of agglutination tests a closer agreement can hardly be expected. Therefore it can be said there is some parallelism between the antibody titers of the serum and cerebrospinal fluid on the one hand and the globulin content of these fluids on the other hand.

These observations have an obvious bearing on the serum therapy of the central nervous system. They show that antibodies do accumulate in the tissue of the brain, spinal cord and cerebrospinal fluid even if the immune serum is not injected into the central nervous sys-

tem; furthermore, that the antibody content of the central nervous system can be estimated by titration of the blood.

It is important to ascertain whether antibodies accumulate in the tissues of the central nervous system at a faster rate when the immune serum is injected into the spinal fluid instead of the peripheral blood stream. This question is being investigated and will be the subject of a subsequent publication.*

In the previous studies it was found that the agglutinin titers of the extracts of the spleen, liver, lung, kidney, uterus and skin are about the same and on the average ten times lower than that of the serum (extract of 1 gm. of organ compared with dilution of 1 cc. of serum). The titer of extracts of the muscles of the leg is lower than those of the other organs examined, varying from 1 to 5 per cent expressed as percentage of the titer of the serum. In contrast to these organs the brain and cord yield extracts that contain antibodies in very low titer, less than 1 per cent of the titer of the serum. (See Chart 1.)

CONCLUSIONS

1. Antibodies can be extracted from the brain and spinal cord of rabbits actively or passively immunized with typhoid bacilli.

2. The titers of the antibodies in the extracts of brain and cord depend upon the titer of the blood serum. In actively immunized rabbits the following numerical relationships exist between the titers of the serum and of these organ extracts: The ratio of the titer of the serum is to the titers of extract of brain and of the spinal cord about as 100 is to 0.8; the titer of the serum is to the titer of the cerebrospinal fluid as 100 is to 0.3. In passively immunized rabbits the titer of the serum is to the titer of brain and spinal-cord extract as 100 is to 0.7.

3. The antibodies recovered from the brain are not due to the presence of blood in it for perfusion of the brain does not reduce its antibody content appreciably.

4. Antibodies penetrate into the spinal fluid from the blood even in the absence of inflammation of the meninges. When the penetration is completed the following numerical relationship exists between the titer of the serum and that of the cerebrospinal fluid: 100 to 0.25.

* More general discussion of the literature will be published in a subsequent paper.

5. The penetration into the cerebrospinal fluid of antibodies injected intravenously proceeds at a slow rate, being completed only several hours after the immune serum has been injected. The penetration of antibodies into the tissue of the brain occurs at a very rapid rate. It is completed within 15 minutes.

6. It is very unlikely that when the immune serum is injected intravenously the antibodies reach the brain tissue by way of the cerebrospinal fluid, for (1) the antibody titer of the cerebrospinal fluid is lower than that of the brain extract, and (2) antibodies penetrate faster into the tissue of the brain than into the cerebrospinal fluid.

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CULTIVATION AND CLASSIFICATION OF "BACTEROIDES," "SYMBIONTS," OR "RICKETTSIAE" OF BLATTELLA GERMANICA

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The writer[1] recently reported the isolation and cultivation of the intracellular microorganism within the large American roach, *Periplaneta americana*, and found that it was a bacterium belonging to the genus *Corynebacterium* (the diphtheroids). Three types could be distinguished on the basis of certain morphological and serological characters. The species was named *Corynebacterium periplanetae* nov. sp. variety *americana*.

It seemed important to determine whether other closely affiliated insects harbor similar intracellular parasites, and whether the parasite can be adapted to artificial media by using the technical procedures already described. The German roach or "croton bug" (*Blattella germanica*) was selected for investigation.

Sections through the eggs, embryos, and body of *Blattella* show the existence of bacteriocytes with an arrangement and morphology similar to those of *Periplaneta*. The cytoplasm of these cells is packed with bacteria-like microorganisms which may be liberated by triturating fresh tissue in physiological salt solution. As in the case of *Periplaneta*, they are non-motile, non-encapsulated bacilli which are not acid-fast but Gram-positive. The microorganisms stain well by Giemsa's method and are seen to be extremely pleomorphic, with equal and unequal division or budding. The bacilli are straight or crescent-shaped, banded or bipolar, and often terminate in a thickening or club. Short, oval, and coccoidal forms are also common, and appear to represent buds produced from the longer rods. Spores have never

[1] Glaser, R. W., *J. Exp. Med.*, 1930, **51**, 59.

been observed. Chains of three units are sometimes seen. The individual units of the bacillary forms have a mean length of approximately 2.5μ with a modal length of 3.2μ. The breadth varies from 0.8 to 1.6μ. The diameter of the coccoidal forms was fairly constant, approximately 0.8μ. The above enumerated facts undoubtedly place the species in the genus *Corynebacterium*.

The procedures for isolating *Corynebacterium periplanetae* free from contaminants and for cultivating the species on dextrose horse blood agar by means of the "spotting technique" have been fully described. The same methods were adopted for the *Blattella* parasite which was isolated and cultivated from the bacteriocytes of six adults and from four embryos removed from egg capsules. Altogether 86 spots were made from these and 10 cultures obtained. Approximately 12 per cent of the attempts succeeded.

The cultivated form corresponds closely to the intracellular parasite. All cultures are non-motile, non-encapsulating, non-sporulating bacilli not acid-fast but Gram-positive. Giemsa's stain shows the same characters previously mentioned, namely, pleomorphism, banding, flexing, with club, oval, and coccoidal forms. In addition cultures on dextrose horse blood agar show many small cocci, diplococci, and *Rickettsia*-like stages. The purity of these cultures can hardly be questioned because they have been repeatedly plated and always yield, in 120 hours, one type of clear, round colony of pinpoint size.

Serological tests with the ten *Blattella* isolations demonstrated the presence of two strains. These strains and their intracellular forms were compared with the *Periplaneta* intracellular parasite and its three cultural types. A morphological differentiation between the intracellular forms was not possible.

Table I represents a series obtained by making 25 measurements for each character. The preparations were fixed in equal parts of absolute alcohol and ether for 15 minutes and stained according to Giemsa's method.

The sizes of the cultural forms reasonably correspond to the sizes of their intracellular counterparts. Slight differences in the mean length of the bacilli occur, but the modal length of the intracellular forms, whether from ova or from mature bacteriocytes, is fairly constant. The cultures show some slight differences from the intracellular forms

and from one another. A perusal of both the mean and modal length of all bacillary forms shows that Type II from *Periplaneta* is the longest and Type III from the same host the shortest. Other differences between the three *Periplaneta* types were pointed out in the earlier work.

Strains I and II from *Blattella* are indistinguishable from one another on the basis of size, but may be segregated from the *Periplaneta* cultural types by the large number of minute coccoidal forms which appear in cultures of the former.

TABLE I

Source of parasites	Length range	Mean length	Modal length	Breadth range	Diameter range of coccoidal forms
	μ	μ	μ	μ	μ
Periplaneta bacteriocytes	2.0–5.0	3.0	3.2	0.5–1.0	0.5–1.0
Blattella "	1.6–4.0	2.5	3.2	0.8–1.6	0.8
Periplaneta ova	0.8–4.8	1.9	3.2	0.5–1.0	0.5–1.0
Blattella "	0.8–4.8	2.4	3.2	0.8–1.2	0.8
Periplaneta cultures:					
Type I	0.5–3.2	1.9	3.2	0.4–0.6	0.5–1.0
" II	1.6–4.8	3.4	4.0	0.5–1.0	0.5–1.0
" III	1.6–3.2	2.1	2.4	0.4–0.6	0.3–0.8
Blattella cultures:					
Strain I	0.8–4.8	2.6	3.2	0.4–0.8	minute–0.8
" II	1.6–4.0	2.8	3.2	0.8–1.2	minute–1.2

The *Blattella* strains cannot be differentiated from those of *Periplaneta* culturally nor on their fermentation reactions to carbohydrates as Table II shows. None of the forms produce gas. They ferment glucose, sucrose, and maltose, but not lactose nor mannite.

Table III represents some agglutination tests with *Blattella* Strains I and II against normal rabbit serum and against rabbit serum immunized against the three *Periplaneta* types.

These tests were performed at the same time as those described in the previous work, so that a comparison is possible. Such a comparison shows that the *Blattella* strains are distinct from those of *Periplaneta*. Serologically Strain II from *Blattella* resembles Type III from *Peri-*

TABLE II

Carbohydrates	Glucose	Lactose	Sucrose	Maltose	Mannite
pH of medium	7.3	7.3	7.3	7.3	7.3
Periplaneta:					
Type I	5.2	7.3	5.2	5.3	7.4
" II	5.2	7.3	5.0	5.3	7.4
" III	5.4	7.2	5.6	5.3	7.3
Blattella:					
Strain I	5.4	7.3	5.5	5.4	7.3
" II	5.1	7.2	5.5	5.3	7.3

TABLE III

Agglutination Tests

Normal rabbit serum and *Periplaneta*, Types I, II, and III immune rabbit sera against the *Blattella* diphtheroid, Strains I and II. Suspensions of microorganisms in 0.20 per cent NaCl solution. Standardization on Gates turbidity scale to 2.0.

Serum immune to	Titer of immunizing culture	Antigen used	Serum dilutions									
			1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1,280	1/2,560	NaCl
Periplaneta:		*Blattella:*										
Type I	1/160	Strain I	−	−	−	−	−	−	−	−	−	−
		" II	++	++	+	+	−	−	−	−	−	−
" II	Clumps spontaneously	Strain I	−	−	−	−	−	−	−	−	−	−
		" II	−	−	−	−	−	−	−	−	−	−
" III	1/10,240	Strain I	−	−	−	−	−	−	−	−	−	−
		" II	C	C	C	+++	++	++	+	+	±	−
Normal serum		Strain I	−	−	−	−	−	−	−	−	−	−
		" II	−	−	−	−	−	−	−	−	−	−

planeta more closely than the other forms. Distinctions also exist between the two *Blattella* forms.

For the species of intracellular diphtheroid found within *Blattella germanica*, the writer would like to propose the name *Corynebacterium blattellae* nov. sp.

SUMMARY AND CONCLUSIONS

In *Blattella germanica*, the German roach or "croton bug," bacteriocytes are found in all individuals of both sexes. These bacteriocytes are scattered throughout the fat tissue and their cytoplasm is filled with microorganisms. Evidence is presented to show that the intracellular parasites are diphtheroidal bacilli. These diphtheroids are transmitted from one generation to another through the ova.

By using a technic previously described, the intracellular parasites were isolated and cultivated from the adult bacteriocytes and from embryos. Two diphtheroidal strains were cultivated with approximately equal frequency. These two strains resemble one another closely enough to be considered a single species but show certain minor differences. The sizes, general morphology, and tinctorial reactions of the two cultures correspond to the intracellular parasites of *Blattella germanica*. They may be distinguished from the three types of *Corynebacterium periplanetae* variety *americana*, previously described. For the species here discussed the name *Corynebacterium blattellae* nov. sp. is proposed.

A CONTRIBUTION TO THE EPIDEMIOLOGY OF SPECIFIC INFECTIOUS CYSTITIS AND PYELONEPHRITIS OF COWS

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Plates 28 and 29

(Received for publication, March 7, 1930)

In previous papers (1,2) we called attention to the presence of a specific disease of the urinary tract of cows which had been known in Europe for many years but had not heretofore been recognized in this country. From our material we concluded that the bladder is first involved with secondary involvement of the ureters and kidneys. A bacterium of the diphtheroid type similar to that obtained by several workers in Europe was readily cultivated from the inflamed portions of the urinary tract. In our experiments when small quantities of pure cultures were introduced into the bladder by way of the urethra, a slowly spreading cystitis resulted but the inflammatory process failed to ascend the ureters. Certain bacteriological and other clinical studies failed to confirm the generally accepted view that the urinary infection was associated with genital disease and we were unable to obtain the specific organism from sources other than the urinary tract. Bacteriological examination of the urine of the bulls of two herds in which the disease was prevalent failed to reveal the specific diphtheroid.

It has been difficult to explain the mode of spread within the herd, although certain experiments in which cows were stabled in close contact with others known to be infected resulted in transmission. It was the practice to curry and brush the cows twice a day and probably the infectious material was repeatedly introduced into the vagina by means of brushes. In some other instances the source of infection was not evident. The propagation of the organism seems to lie within

the urinary tract of infected cows. Under usual herd conditions transmission thus becomes a difficult matter, since septic material is not usually brushed into the vagina. Some natural means of propagation must necessarily exist and our problem concerned itself with this question.

Dr. Theobald Smith (3), in his examination of the urine of calves, frequently encountered diphtheroids in large numbers, and he has suggested that they might be of significance. It was with the idea of correlating these organisms with those from bladder infections of adult cows that the following studies were made.

Method

In the main the calves were drawn from one herd from which the bulk of our original material had been obtained. A group of the calves were brought to the Institute and used for other experiments, and at autopsy the genito-urinary tract was turned over to us for examination. These were considerably older than the calves from which we obtained material at the abattoir. The calves slaughtered at the Institute varied from 3 weeks to 2 months of age, while those killed at the abattoir were from a few days to 2 weeks old. In a few instances we obtained material from heifers 3 to 4 months old, but most of the material came from males.

The genito-urinary tract was removed in one piece and the whole tract seared. The urine was withdrawn from the bladder and the tract incised from the bladder to the sheath. Cultures were prepared from the bladder mucosa, the upper, middle and lower portions of the urethra, and the sheath, by scraping the mucosa vigorously with a curette and streaking the surface of blood agar plates with the scrapings. The plate cultures were incubated for 48 hours and suggestive colonies picked for inoculation into other media. When material from female calves was examined, scrapings from the bladder, urethra and vagina were made. The urine obtained from the bladder was centrifuged and plate cultures prepared from the sediment. The urinary tracts of 34 calves were examined.

The cultures which conformed in general to the diphtheroidal type were propagated, and, if regarded of sufficient interest, studied on differential media. In this way it was found that several distinct cultural groups existed among the strains. This has been brought out in Table I.

Table I indicates that the diphtheroids fall into 5 cultural groups. It has been shown that the bacterium associated with specific cystitis and pyelonephritis ferments dextrose and fails to ferment the other substances. It produces enough alkali in litmus milk to turn the litmus a deep purple and finally the medium becomes sufficiently alka-

TABLE I

The Cultural Characters of Diphtheroids Isolated from the Urinary Tract of Calves

Group	No. of strains	No. of calves from which isolated	Final hydrogen ion concentration in					Effect on milk	Effect on gelatin	Chromogenesis on potato
			Dextrose	Lactose	Saccharose	Maltose	Mannitol			
I	25	12	4.9–5.3	7.4–8.0	7.4–8.0	7.4–8.0	7.4–8.0	Becomes alkaline; casein dissolved	Not liquefied	Varies from white to faint cream to orange
II	44	18	5.4–5.8	7.6–8.0	6.6–5.0	6.6–5.2	7.6–8.0	"	"	Grayish yellow to orange
III	11	8	5.6	8.0	8.0	6.0	8.0	"	"	Orange
IV	8	3	5.2–5.4	8.0	7.6–8.0	6.8	7.6–8.0	"	"	Cream to orange
V	40	16	7.4–8.0	7.4–8.0	7.4–8.0	7.4–8.0	7.4–8.0	Remains unchanged or becomes alkaline; casein dissolved	"	Yellow to orange

line to dissolve the casein. From Table I it is evident that we succeeded in isolating a large number of strains of proper morphology which possessed the biochemic properties of the strains isolated from the disease. The table also indicates that other types of diphtheroids occur frequently in the urinary tract. Some of them when freshly isolated are readily distinguishable morphologically from those of the first group. Their biochemic features lead to a ready differentiation. It should be stated that all types were non-motile and stained intensely by the Gram method.

The distribution of the various cultural types throughout the genito-urinary tract is of interest and is shown in Table II.

TABLE II

The Distribution of the Various Types in the Genito-Urinary Tract

Group	Isolations from				
	Sheath	Urethra	Bladder	Urine	Vagina
I	16	5	0	3	1
II	7	28	8	1	0
III	1	4	5	1	0
IV	3	4	1	0	0
V	10	11	10	6	3

Cultural Type I, which we regarded as of considerable significance, seems to be confined largely to the sheath. Its entire absence from the bladder is noteworthy although certain strains were recovered from the lower portion of the urethra. On the other hand, the second cultural group seems to live largely in the urethra and bladder, since 36 of 44 strains were isolated from these regions. Group V occurs throughout the urinary tract and has been isolated from the vagina. The other two groups contain such small numbers that their distribution seems to have little meaning.

Since the first group possessed all the morphological and cultural characters manifested by the organism associated with the specific infection in the adults, further correlations were manifestly desirable. With this in view a number of strains were chosen for further study. Rabbits were immunized with cultures isolated from the kidneys of

TABLE III

The Agglutination Affinities of the Group I Strains Obtained from the Urinary Tract of Calves

Culture	Dilutions of serum of rabbit immunized with culture from spontaneous case							Dilutions of serum of rabbit immunized with culture from Calf 1474 sheath							Control
	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:5	1:10	1:20	1:40	1:80	1:160	1:320	
Cow 1132 (left kidney)	C*	C	C	++++	+++	++	++	C	C	C	++	++	+	+	–
Calf 1474 (sheath)...	C	C	C	C	++++	+++	++	C	C	C	C	++++	+++	±	–
" 1494 " ...	C	C	++++	++++	++	+	–	C	C	+++	++	+	–	–	–
" 1539 (urethra)...	C	C	C	B	B	B	B	C	C	C	B	B	B	B	B
Cow R 418 (urine)...	C	C	C	++++	+++	++	++	C	C	++++	+++	++	+	+	–
Calf 1533 (sheath)...	C	C	C	++++	+++	+++	++	C	C	++	++	++	++	++	–
" 305 (urethra)...	C	C	+++	B	B	B	B	C	C	+++	B	B	B	B	B
" 1540 (urine).....	C	C	C	++++	+++	++	+	C	C	++++	++	++	++	++	+
" 306 (urethra)...	C	C	+++	+++	+++	+	+	C	C	C	+++	++	++	++	+
" 1480 (urine).....	C	C	C	+++	+++	++	++	C	C	C	+++	++	+	±	–
" 303 (sheath)....	C	C	+++	++	+	+	+	C	C	+++	++	++	++	+	–
" 307 "	+++	C	C	+++	–	–	–	C	C	+++	+	–	–	–	–
" 1480 "	++++	+++	+	B	B	B	B	C	C	++	+	B	B	B	B
" A "	C	C	B	B	B	B	B	C	C	+++	B	B	B	B	B
Cow D. M. (urine)....	C	C	C	C	C	C	+++	C	C	C	++++	+++	+++	++	–
Calf 1531 (sheath)....	C	C	+++	+	–	–	–	C	C	++	+	±	–	–	–
" 1522 (urine).....	C	C	C	C	+++	++	+	C	C	C	+++	++	+	+	+

* C = complete. The quantity of agglutination has been recorded as +, a definite but slight clumping, to ++++, a heavy clumping without clearing. B = spontaneous precipitation in the form of a button at the bottom of the tube.

spontaneous cases and with certain typical strains from calves. When sera with sufficient agglutinin were obtained all strains were tested. That these possessed agglutination affinities similar to those of the organisms isolated from the kidneys of adults in spontaneous infections is shown in Table III.

On the whole it can be said that the strains resembling the etiological type behave in a manner similar to those isolated from spontaneous cases in cows. The serum from a typical calf strain is capable of agglutinating those from the cows and *vice versa*.

TABLE IV

The Effect of Reciprocal Absorption on Agglutination

Culture	Serum dilutions of culture Cow 1132 serum absorbed with culture Calf 1474						Serum dilutions of culture Calf 1474 serum absorbed with culture Cow 1132						Control
	1:10	1:20	1:40	1:80	1:160	1:320	1:10	1:20	1:40	1:80	1:160	1:320	
Cow 1132 (kidney)...	±	−	−	−	−	−	+	±	−	−	−	−	−
Calf 1474 (sheath)....	−	−	−	−	−	−	±	−	−	−	−	−	−
" 1494 "	+	+	−	−	−	−	−	−	−	−	−	−	−
" 1539 (urethra)...	+	−	−	−	−	−	−	−	−	−	−	−	−
Cow 418 (urine).....	+	±	−	−	−	−	+	−	−	−	−	−	−
Calf 1533 (sheath)....	+	±	±	±	±	±	+	+	±	±	±	±	±
" 1540 (urine).....	±	±	±	±	±	±	±	±	±	±	±	±	±
" 306 (urethra)...	+	±	±	±	±	±	++	±	±	±	±	±	±
" 1480 (urine).....	+	−	−	−	−	−	±	−	−	−	−	−	−
" 303 (sheath)....	±	±	±	±	±	±	+	±	±	±	±	±	±
" 1480 "	−	−	−	−	−	−	−	−	−	−	−	−	−
" A "	−	−	−	−	−	−	−	−	−	−	−	−	−
Cow D. M. (urine)...	±	−	−	−	−	−	+	+	±	±	±	−	−
Calf 1531 (sheath)....	−	−	−	−	−	−	−	−	−	−	−	−	−
" 1522 (urine).....	±	±	−	−	−	−	±	±	−	−	−	−	−

In the course of the correlation it seemed advisable to test the antigenic specificity further. With this in mind, the serum produced by immunization with the strain obtained from a cow's kidney was absorbed with the culture obtained from the sheath of Calf 1474 and the other serum was absorbed with the culture isolated from the kidney. The absorbed sera were then tested with the various strains as shown in Table IV.

The results of the tests of the sera after absorption indicate that

a typical strain obtained from the sheath of a healthy calf is capable of absorbing practically all the agglutinin from the antiserum specific for the etiological strains. The same held true when the serum produced by immunization with one of the calf strains was absorbed with a culture from an adult infection. The cultural and immunological findings support the view that the organisms are similar.

The calves from which cultures were obtained were apparently normal. This brought up the question of pathogenicity. It is known that freshly isolated cultures when injected into rabbits or guinea pigs in considerable quantities fail to produce lesions. Our previous experiments indicated that small quantities of culture from either bladder or kidney infections when introduced into the cow's bladder by means of a catheter gave rise to a slowly progressive persistent cystitis. The most important test of identity seemed to us to be that of the pathogenic properties of the strains. Four cows were available for the tests and these were injected with various cultures. The details are given in the following protocols. The urine from all was examined twice at intervals before the injections were made. Cultures failed to show significant diphtheroids and the urine was free from albumin and other abnormal products.

On April 22, 1929, 4 cows were inoculated intra-urethrally with 3 cc. of 48 hour broth cultures, as follows: Cow 1517, culture from the urine in spontaneous infection ot Cow R 418. Cow 1520, culture from the lower urethra of Calf 1539. Cow 1530, culture from the sheath of Calf 1539. Cow 1559, culture from the sheath of Calf 1480.

One week later the urine of all was examined. The urinary sediment of all contained diphtheroids similar in morphology and cultural characters to those inoculated. Six days after the first examination specimens were again examined. The urine from Cows 1517, 1520, and 1530 showed little abnormal; that from Cow 1559 was turbid and gave a strong reaction for albumin. One week later the urine from all 4 cows was reexamined with the same results. The diphtheroids had disappeared from the urinary sediment of 1517, 1520, and 1530. The specimen from 1559 contained blood, gave a strong albumin reaction, and was rich in red cells, clots and diphtheroids. It was at this time that the first urethral hemorrhage was observed. Urination became more frequent and considerable straining following micturation, often accompanied by the passage of bright red blood. A sample of urine obtained 3 months after the inoculation revealed blood, mucus, and large numbers of diphtheroids.

Cow 1559 was slaughtered at an abattoir 6 months and 21 days after inoculation.

The whole genito-urinary tract was brought to the laboratory for examination. The bladder was contracted, its walls thickened, and the vessels of the subserosae dilated. The whole mucosa was congested, swollen and wrinkled, and scattered over its surface there were many irregularly rounded raised hemorrhages. On the floor of the bladder were a number of irregular, slightly raised, flattened, adherent patches which were removed with difficulty, exposing a raw, weeping surface. The mucosa between and about the outlets of the ureters appeared as a wrinkled, greatly swollen, reddened, spongy mass. Both ureters were dilated and the walls markedly thickened. The right was almost half again as large as the left (Fig. 1). The mucosa of both appeared edematous and was streaked here and there with longitudinal red lines. Portions of two lobes of the right kidney contained irregularly rounded, grayish white, homogeneous areas which involved only the cortex. Several irregular, sharply circumscribed accumulations of yellowish green caseous material were present at the tips of the papillae (Fig. 1). The areas varied from 1 to 2 mm. in diameter to nodules as large as 1 cm. (Fig. 2). The caseous material shelled out easily revealing a glistening capsule. Films from the caseous areas revealed enormous numbers of diphtheroids. Portions of the mucosa of the pelvis were swollen and reddened (Fig. 1). The mucosa of the ureter where it left the kidney revealed a similar process (Fig. 1). In addition to these findings a cyst which protruded from the vaginal wall was noted. Diphtheroids were readily obtained from the urine at autopsy. Films from the pelvic abscesses showed diphtheroids in enormous numbers to the exclusion of other forms. Cultures developed only diphtheroids. The left kidney revealed no abnormalities.

Pieces of the bladder, ureter and right kidney fixed in Zenker's fluid revealed well marked changes. Microscopic examination of sections of the bladder showed a variety of lesions. The mucosa in one area was largely intact but the submucosa was greatly thickened, edema was pronounced, accumulations of round cells and hyperplasia of the connective tissue accompanied by capillary proliferation were remarkable. In other portions little of the mucosa remained except for a few streamers of degenerated cells. The exposed surface consisted of a richly cellular submucosa composed of proliferating connective tissue containing many vessels and heavily infiltrated with round cells. The left ureter presented little abnormal except edema of the submucosa and proliferation of the submucous connective tissue. The mucosa of the right ureter revealed degenerative changes and some exfoliation. The deeper portions of the mucosa were invaded by round cells. The submucosa was edematous and rich in capillaries as well as moderately infiltrated with round cells.

The left kidney presented no serious derangement. The section from the right revealed little abnormal in the cortex. The medulla presented no abnormality until close to the papillus, where some of the tubules revealed exfoliation of the mucosa. There was a moderate engorgement of the capillaries. Nearer the pelvis the desquamation was more marked and the vascular engorgement

more pronounced. About the tip of the papillus and extending a uniform depth into the kidney a well defined lesion was observed. On the whole the tubular outlines were well preserved but the mucosa had disappeared and the lumina were plugged with homogeneous material. Hemorrhages into the tubules were not infrequent. Under lower magnifications several deep blue staining, clear cut masses lying close to the papilla and having the outlines of large tubules were readily discerned (Fig. 3). These when sufficiently magnified proved to be tubules of which only the basement membrane remained, and the blue staining areas were composed of densely packed masses of diphtheroids (Fig. 4). On the whole the lesion impressed us as being relatively recent and was characterized by its sharp definition and its relative freedom from cellular reaction. The remarkable growth of the causative organism within the tubules with consequent interference with the neighboring circulation and the absorption of bacterial products might well account for its character.

The other 3 cows were killed 24, 39, and 53 days after inoculation. The bladders and kidneys failed to show abnormalities and the organisms could not be recovered from the urine.

It will be seen from the protocols that two of the three cows inoculated with cultures obtained from the lower urinary tract of calves developed only transient infections. The inoculation in the third instance (Cow 1559) produced cystitis, pyelitis and pyelonephritis. The cow injected intra-urethrally with culture from the kidney of a spontaneous infection developed only transient cystitis.

DISCUSSION

The findings indicate that the lower genito-urinary tract of male calves may harbor an organism similar in morphology, cultural characters and certain immunological properties to the organism encountered in this country and Europe in spontaneous infections of the bladder, ureter and pelvic portions of the kidneys of cows. The organism has been found in the sheath, urine, and lower portions of the urethra in 12 of 34 calves examined. By far the greatest number of strains were obtained from the sheath. The mucosa of the sheath and the retained urine habor bacteria in enormous numbers and the diphtheroid seems to make up but a small proportion of the organisms present.

The findings are significant from an epidemiological standpoint. As we have pointed out, the difficulty in tracing infection from animal to animal within the herd has been great. It seems probable that many cases may really originate as infections during early life. The

organisms are able to maintain themselves on the mucous membranes of the lower urinary tract where little damage is done, but by a gradual, ascending growth they may finally reach the upper urethra and bladder. Where the tube is short as in the female the supposition is reasonable; and in this sex additional complications, such as pressure on the bladder, albuminuria, etc., may act to quicken the invasive process. Unfortunately our material contained only a few female calves; but from one of these we obtained from the vagina in the region of the urethral orifice a culture indistinguishable culturally and immunologically from the pathogenic type. The source of the fairly frequent sheath infestation is not easily traced. In one instance a calf born of an infected mother was examined and the organism recovered from the sheath. The same organism was readily cultivated from the mother's urine and in all probability the sheath was directly inoculated during parturition. In five other instances the urine of the mothers of calves in which we had cultivated the diphtheroid from the sheath was examined with negative results.

The brief citation of a spontaneous infection in a 12 day old male calf serves as evidence that infection may begin in early life.

The genito-urinary tract was obtained immediately after slaughter. The kidneys presented no gross abnormalities. The bladder walls were greatly thickened and the mucosa swollen and edematous. Hemorrhages varying in size from minute points to considerable extravasations were scattered over the mucosa. The urine within the bladder was turbid and contained blood. The urinary sediment was rich in red cells, round cells, leucocytes, and diphtheroids. Typical diphtheroids were obtained in pure culture. Microscopic examination of fixed and stained material from the bladder revealed the usual type of cystitis encountered in adult infections of this kind.

Certain facts (2) indicate that the sheath of the adult male does not contain many organisms of the etiological type, since we failed to cultivate them from naturally passed urine in 11 instances, although organisms similar to those of Groups II and IV were readily grown.

From the experiment in which 3 cows were artificially infected with strains from calves and only 1 developed the disease, it might be suggested that certain of the calf strains are not pathogenic. It is true that all the cultures employed in the injection had been under artificial cultivation for several months and that that from the spontaneous

case was isolated 2 years before the inoculation. The strain which produced the infection in the inoculated cow had been isolated about 1 year before it was used. On the other hand, as a control procedure, a cow was inoculated with a culture obtained from the kidney of a severe case but this gave rise only to transient cystitis. From this it can be argued that the resistance of certain cows might explain our results.

We have produced cystitis in a fair proportion of inoculations, but the disease has thus far been confined to the bladder. In the instance of the cow which we regard as most susceptible (1559) culture introduced into the bladder produced the local process and later reached the pelvis of one kidney by way of the ureter. The experimental evidence indicates that under favorable conditions diphtheroids introduced into the bladder may produce cystitis, pyelitis and pyelonephritis.

SUMMARY

Bacteriological examination of the genito-urinary tract of calves originating in a herd in which infectious cystitis and pyelonephritis exists among the cows, revealed a variety of cultural types of diphtheroids. Of these types, one obtained from a considerable number of the calves resembled in morphology and cultural characters the organism cultivated from the actual cases of the disease. This group had agglutination affinities like those of the organism mentioned and was capable of absorbing agglutinin from antiserum specific for it. When three cows were inoculated intra-urethrally with cultures isolated from the sheaths of calves, two developed transient infections and the other a severe prolonged cystitis and pyelonephritis.

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EXPLANATION OF PLATES

PLATE 28

FIG. 1. The right kidney of Cow 1559 which had been inoculated intra-urethrally with culture obtained from the sheath of a normal calf. Note edema and congestion of the pelvic mucosa, the size and appearance of the ureter. The arrows indicate the location of small abscesses. About $\frac{2}{3}$ actual size.

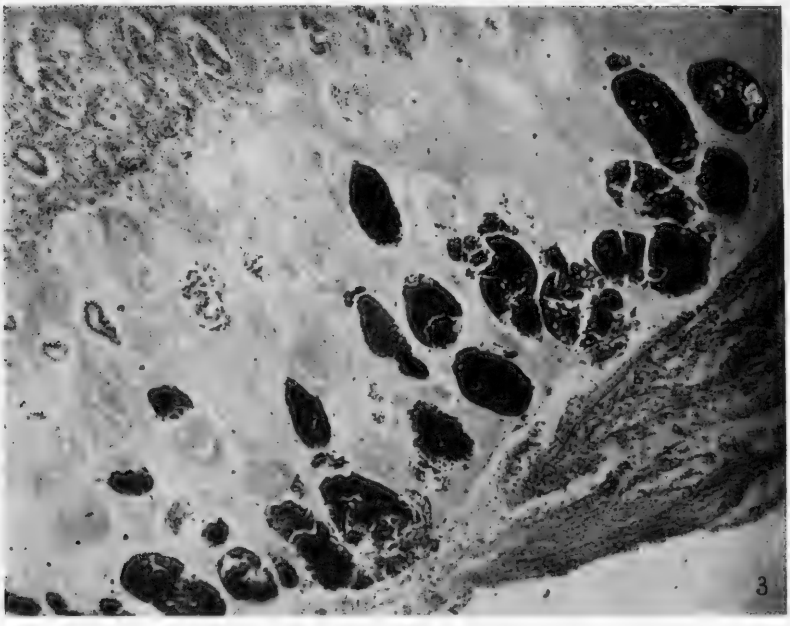
FIG. 2. The largest abscess encountered in another portion of the right kidney. × about $1\frac{1}{4}$.

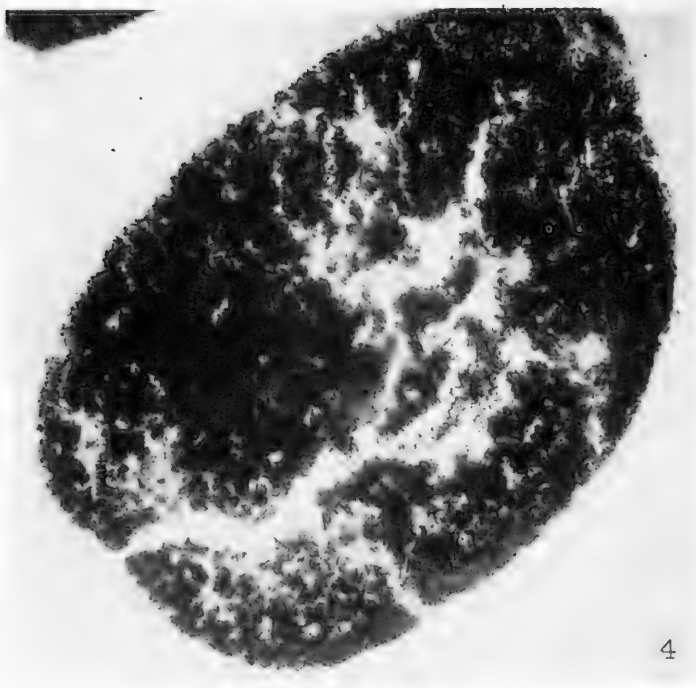
PLATE 29

FIG. 3. Section of the right kidney of Cow 1559 showing an early lesion deep within the medulla. Note the degenerative changes and the intensely black tubules due to deeply stained bacterial masses within them. Zenker's fixation; eosin and methylene blue stain. ×90.

FIG. 4. A cast composed of tightly packed masses of the diphtheroid from a single tubule from the same preparation. ×660.

(Jones and Little: Cystitis and pyelonephritis of cows)





(Jones and Little: Cystitis and pyelonephritis of cows)

OF MICH

THE EFFECT OF CATHODE RAYS UPON CERTAIN BACTERIA

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Plate 30

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A study of the rate of killing of bacteria by cathode rays is a step towards finding out what changes these radiations and X-rays bring about in cells. The great interest to biology of X-rays, and probably also of cathode rays, lies not in the fact that they are lethal, but rather in their ability to produce permanent and inheritable changes in cell properties. Nevertheless, because they are deadly it is obvious that an understanding of the conditions under which cells are killed is a necessary preliminary to any thorough-going study of how they are changed. Bacteria have been used in the following experiments partly because they are, on the whole, the simplest cells to work with, partly because much has already been done on the lethal action of X-rays upon them, and partly because through their small size they provide the best introduction to the examination of the effects of these radiations upon the still smaller and imperfectly understood viruses.

Experimental Procedure

In the experiments to be described in this paper, single bacteria spread upon an agar plate are bombarded with a known number of cathode rays and, after incubation, the number of survivors is determined through counts of the colonies of bacteria. From these data and various well known physical characteristics both of the rays and of the bacteria, a satisfactory picture can be gained of many details of the destruction of these cells.

The cathode rays were obtained from a Coolidge type electron tube[1] leased from the General Electric Company. This tube is essentially a large hot cathode X-ray tube with a hollow anode covered by thin metal foil. Many electrons proceeding from the cathode pass through this anode and foil and, emerging into the air, constitute the cathode ray beam. The velocity of these electrons and, in general, the transparency of matter to them increases with the voltage applied to the tube. For these experiments the voltage was approximately 155 K.V. The velocity of the emitted electrons is of the order of 0.8 of the velocity of light.

The absorption of an electron in inanimate matter is attended by the release of a large number of ions within a very small volume. A 150 K.V. electron will liberate about 10^4 ions within less than 0.001 mm.[3] It is natural and in accord with existing knowledge to associate the changes brought about in a bacterium by cathode rays with the absorption of electrons and the consequent shower of ions freed within it. Together with this ionic shower, X-rays are emitted as a consequence of electron absorption. The passage of cathode rays through air is likewise attended by the formation of much ozone and it might be argued that the observed destructive action on bacteria was due either to these X-rays or to ozone. We have ascertained by suitable experiments that this is not the case.

Several conditions must be fulfilled if a statistical analysis of the killing of bacteria by cathode rays is to have any physical significance. In the first place, the absorption of these rays is so great that the bacteria must be exposed upon the surface of an agar plate rather than held in a suspension if the dose they receive is to be satisfactorily measured. It is likewise necessary that they be spread upon this surface with sufficient uniformity so that colony counts in selected standard areas can be taken as measures of the numbers of irradiated bacteria. Not only must the bacteria be spread as single organisms, but their multiplication must be prevented until irradiation is completed. This is evident when it is realized that if several organisms are associated together in a clump, a single survivor will produce a countable colony. Many experimental procedures were tried with several types of bacteria before one was found which gave a satisfactory spread of single organisms. The highly motile *B. aertryke* gave excellent results with 200 to 300 organisms per square inch and almost as satisfactory data were provided by *B. coli* in somewhat greater dilution. Repeated trials, however, failed to produce as good spreads of single organisms of *Staphylococcus aureus*. This bacterium has frequently been used in

[1] Coolidge, W. D., *J. Franklin Inst.*, 1926, **202**, 693.

studies of the action of X- and ultraviolet rays. Our experience makes it seem probable that single cell spreads were not ordinarily obtained in these experiments.

The bacteria were prepared for irradiation in the following way. Broth cultures, from standards provided by Dr. L. T. Webster of this Institute, were carried for at least 3 days through daily transplants before use. The final 20-hour old tube was diluted an hour before irradiation using either physiological salt or Locke's solution. This dilute suspension, so chosen as to give the desired number of bacteria per plate, was kept in an ice bath until actually used in order to prevent

TABLE I

Survival Ratios in Typical Experiments

Time	Survival ratios				
	(1) *B. coli*	(2) *B. aert.*	(3) *B. aert.*	(4) *B. aert.*	(5) *S. aureus*
4 sec.	0.698	0.562	—	—	0.853
5 "	.610	.417	0.699	0.671	.745
8 "	.455	.310	.602	.612	.566
12 "	.311	.258	—	—	.341
14 "	—	—	.481	.372	—
16 "	.204	.174	—	—	.248
20 "	.061	.151	.321	.264	.169
24 "	.050	.103	—	—	.168
28 "	.026	.055	—	—	.205
Electrons incident per cm.²/sec.	1.65×10^9	—	0.85×10^9	0.96×10^9	2.99×10^9

cell multiplication. In seeding, 1 cc. of this suspension was run evenly over the agar surface of a poured 10 cm. Petri dish. After vigorous shaking to remove the excess fluid, the plate was allowed to drain 10 minutes and its edges wiped free from adhering liquid. A known area was then immediately irradiated and another marked as standard. After incubation over night, counts were made of the colonies developing in each region. Approximately 100 plates were irradiated for each experiment. In some experiments the plates themselves were refrigerated before being inoculated. This proved to be unnecessary and had the disadvantage that the attendant condensation of moisture on the agar surface often destroyed the bacterial distribution.

The plate ready for irradiation was placed in the holder C of Fig. 1 and its agar surface pressed against the cutting edge of the tube A. This tube is divided

lengthwise into two hemicylinders by a brass and lead partition. The electrons stream through the lower of these halves from the tube window, D, and strike the surface of the agar pressed into the cutting edge. The upper half, sealed with lead to exclude the electrons, provides a standard adjacent to the irradiated surface and of equal area. Cutting through the agar with the tube A has the double advantage of defining sharply the irradiated area and of marking permanently both it and the standard. The tube end was sterilized between irradiations by wiping with alcohol. The time of irradiation was controlled by operating a heavy lead shutter shown at B. In counting the number of electrons emerging from the tube, the measuring device to be described later replaced the plate, other conditions being the same as during irradiation.

In each experiment involving about 100 plates eight different times of exposure were commonly used and 10 or more plates were irradiated for each of these times. The ratios of the colony counts made after incubation upon their irradiated and standard areas were averaged for each time of exposure to give the typical results of Table I.

Analysis

These data can be analyzed with the help of the following considerations from elementary probability theory. If a' is the probability that an event will take place, by definition the probability of its not happening is $b' = 1 - a'$. Furthermore, if c' is the probability that some other event will occur, then the probability that a' and c' will happen together is $a'c'$. In the experiments of this paper many electrons are being shot at a few bacteria. If a is the probability that an electron will hit a bacterium and n is the number of electrons shot at it, the probability that one of these electrons will hit and that every other electron will miss it is

$$a\,(1 - a)^{n-1}$$

Since any one of the n electrons may hit, the probability of striking a bombarded bacterium only once is

$$P_1 = na\,(1 - a)^{n-1}$$

Similarly, the probability of hitting a bacterium by each of two selected electrons is a^2 and the probability of doing it only twice and by these two out of the n electrons is

$$a^2(1 - a)^{n-2}$$

If there are ${}_nC_2$ ways of combining two out of n electrons, the probability that a bacterium is hit twice and only twice is

$$P_2 = {}_nC_2\,a^2\,(1 - a)^{n-2} = \frac{n\,(n-1)}{\underline{|2}}\,a^2\,(1 - a)^{n-2}$$

In the same way, the probability that a bacterium will be struck r out of n possible times is

$$P_r = {}_nC_r\, a^r\, (1 - a)^{n-r} = \frac{n\,(n-1)\,(n-2)\ldots(n-r+1)}{\lfloor\underline{r}} a^r\,(1-a)^{n-r}$$

The number of electrons striking the irradiated surface per second is of the order of 10^{10}. The number n, therefore, may be expected to be very large compared to r, the number of hits. If this is true, then

$$(1 - a)^{n-r} \overset{ca}{=} (1 - a)^n$$

and

$$n\,(n-1)\,(n-2)\ldots(n-r+1) \overset{ca}{=} n^r$$

and

$$P_r \overset{ca}{=} \frac{1}{\lfloor\underline{r}} a^r n^r\,(1-a)^n$$

Expanding $(1\text{-}a)^n$ by the binomial theorem gives

$$(1 - a)^n = 1 - an + \frac{n(n-1)}{\lfloor\underline{2}} a^2 - \frac{n(n-1)\,(n-2)}{\lfloor\underline{3}} a^3 + \ldots\ldots$$

$$\overset{ca}{=} 1 - an + \frac{1}{\lfloor\underline{2}}(an)^2 - \frac{1}{\lfloor\underline{3}}(an)^3 + \ldots\ldots\ldots\ldots$$

By MacLaurin's formula this is

$$(1 - a)^n \overset{ca}{=} e^{-an}$$

In this way we arrive at the Poisson law[2]

$$P_r = \frac{1}{\lfloor\underline{r}}(an)^r\, e^{-an} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

in which an, the average number of hits, can be calculated from the results of irradiation. If one hit is enough to kill a bacterium, the survival ratios as given

---

[2] This expression or its equivalent has been used several times in studies of the effects of X-rays on cells. See for instance Condon, E. U., and Terrill, H. M., *J. Cancer Res.*, 1927, **11**, 324; Crowther. J. A., *Proc. Roy. Soc.*, 1926, B **100**, 390; Holweck, F., *Compt. rend.*, 1929, **188**, 197.

in Table I will be expressed by the probability that a bacterium will be missed every time. As the foregoing considerations have shown, this is

$$(1 - a)^n \overset{ca}{=} e^{-an}$$

In other words

$$\frac{A_1}{A_0} = \text{survival ratio} = e^{-an} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

If a bacterium could withstand one electron but were destroyed by the second, the survivors would be those which either escaped or were hit once, so that

$$\frac{A_2}{A_0} = e^{-an} + an\, e^{-an} = e^{-an}\,(1 + an) \ldots\ldots\ldots\ldots\ldots\ldots\ldots (3)$$

Similarly, if r hits are needed

$$\frac{A_r}{A_0} = e^{-an} + an\, e^{-an} + \frac{1}{2}(an)^2 e^{-an} + \ldots\ldots\ldots$$

$$\ldots\ldots \frac{1}{\lfloor r - 1} (an)^{r-1} e^{-an}$$

$$= e^{-an}\left[1 + an + \frac{1}{2}(an)^2 + \ldots\ldots \frac{1}{\lfloor r - 1} (an)^{r-1}\right]$$

Standardization

The average number of hits can readily be calculated from a knowledge of (1) the area of the irradiated surface, (2) the dimensions of a bacterium, (3) the number of electrons striking the irradiated surface and (4) the absorption coefficient of electrons in the bacterium.

The counting of the number of electrons under the conditions of these experiments cannot accurately be carried out.[3] This is due partly to the enormous ionization produced in the air through which a cathode ray beam passes, partly to the fact that the absorption of the radiation from the tube in small thicknesses of matter is not linear and is accompanied by a considerable diffusion of the transmitted beam. Reproducible results which seem to be fairly accurate have, however, been obtained by the use of a counting chamber which consists of a

[3] See also Thaller, R., *Physikal. Zeit.*, 1928, **29**, 841.

brass disk enclosed, except for a $\frac{5}{16}$ inch hole on one side, by a grounded and air-tight brass sheath. This sheath is a cylinder 2 inches in diameter and 1 inch high and is provided with a port for evacuation. The central disk is supported by a thin rod which, mounted in amber, provides electrical connection with one of the galvanometer leads. The current produced by the flow of electrons from the collecting plate to ground through a sensitive galvanometer has been taken to measure the number of electrons in the beam. This outfit has been placed directly behind the cutting tube A of Fig. 1 in the position of the Petri dish during irradiation and counts have been made at the conclusion of individual experiments. In use, grounded shielding tubes protect the emerging electrode of the collecting plate and the galvanometer leads pass through grounded copper sheathing. The shutter and cutting tube were grounded during both the irradiations and the counting measurements. Standardizations have been carried out (1) with the opening free and (2) with it covered by 0.001 inch aluminum foil and with the chamber evacuated. The fact that the two measurements were substantially identical after allowance had been made for absorption in the foil indicates that the potential developed between the collecting plate and the grounded case was insufficient to interfere seriously with the accuracy of measurement by drawing ions from the air in the chamber. The principal errors in the electron counts probably arise, therefore, from the reflection of electrons by the collecting disk and from inhomogeneity in the cathode ray stream over the irradiated area. No effort has been made to introduce corrections for reflection but shifts of the collecting chamber over the area of irradiation indicated that at the distance of the plate from the mouth of the tube, inhomogeneity of beam was unimportant.

Measured galvanometer currents were corrected for the relative areas of the irradiated surface and the opening in the collecting chamber and were expressed in electrostatic units. Taking one E. S. U. as equivalent to 0.21×10^{10} electrons, the numbers of electrons incident per cm.^{2} of irradiated surface are recorded in Table I.

The absorption of electrons in the bacteria themselves can scarcely be measured. Because this absorption, like that of X-rays, is not a function of the state of chemical combination it can, however, be estimated from measurements upon cellophane as a substance com-

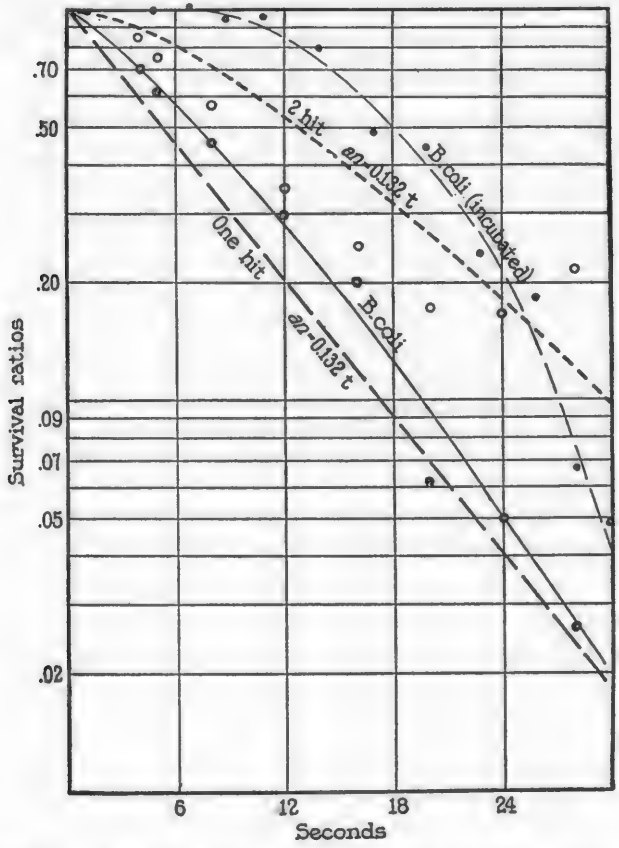
posed only of light atoms and having approximately the density of bacteria. It is well known from studies of β-rays that the absorption of a stream of electrons possessed of different velocities rarely follows any simple law.[4] Measurements of the absorption of the cathode ray beam used in these experiments in successive thicknesses of cellophane lie on a curve intermediate between a straight line and a simple exponential curve. Considering the extrapolation involved in estimating the absorption in a single bacterium from the absorption in a sheet of cellophane (0.0009 inch thick), it has not seemed significant to get more than the order of magnitude of this absorption by a linear interpolation from measureable thicknesses. This procedure has led to the conclusion that there will be an absorption of about 0.008 of the electrons striking either a cylindrical bacterium 0.5μ in diameter and 2μ long or a coccus having a radius of 0.4μ.

DISCUSSION

Whether more than one electron is needed to kill a bacterium can be told from the shape of the survival curves. Expression (2), being a simple exponential, will give a straight line as a graph on semilogarithmic paper. Plotted in this way, the equations for multiple hits yield curves which depart further from a straight line the larger the number of requisite absorptions (Text-fig. 1). Some of the data of Table I are shown graphically in Text-figs. 1 and 2. Within experimental limit the results of the best measurements lie on straight lines. From this fact alone it is to be concluded that the absorption of one electron is sufficient to kill a bacterium. The best obtainable data with *Staphylococcus aureus* depart appreciably from a straight line through the origin. This departure is to be expected, however, for microscopic examination shows that even great dilutions contain many clusters of two or more individuals. The effect of a short incubation of *B. coli* between spreading and irradiation is shown by the top curve of Text-fig. 1. Cell multiplication which has taken place gives a curve resembling but more extreme than that from the coccus.

[4] *Cf.* for instance Rutherford, E., Radioactive Substances and their Radiations, London, 1913, Chapter V.

Although one hit kills, it is not necessarily true that every one does so. In Experiment (1) of Table I, 1.17×10^{10} electrons per second



TEXT-FIG. 1. The black circles of this figure are the survival ratios of Experiment (1) of Table I; the open circles refer to Experiment (5) with staphylococci.

are incident upon the irradiated area of 7.07 cm.^{2} The average number of electrons absorbed per second by a cylindrical bacillus $0.5\mu \times 2\ \mu$ is then

$$\frac{1.17 \times 10^{10} \times 1.00 \times 10^{-8} \text{ cm.}^2 \times 0.008}{7.07 \text{ cm.}^2} = 0.132$$

and Equation (2) as applied to this experiment becomes

$$\frac{A_1}{A_0} = e^{-0.132\,t}$$

The graph of this expression in Text- fig. 1 lies close to, though somewhat below, the experimental survival ratios. The curve of the corresponding equation if two hits are needed to kill (Equation 3) is shown as the thick dotted line of the same figure. Similar relations prevail in the other standardized experiments. The average number

TABLE II

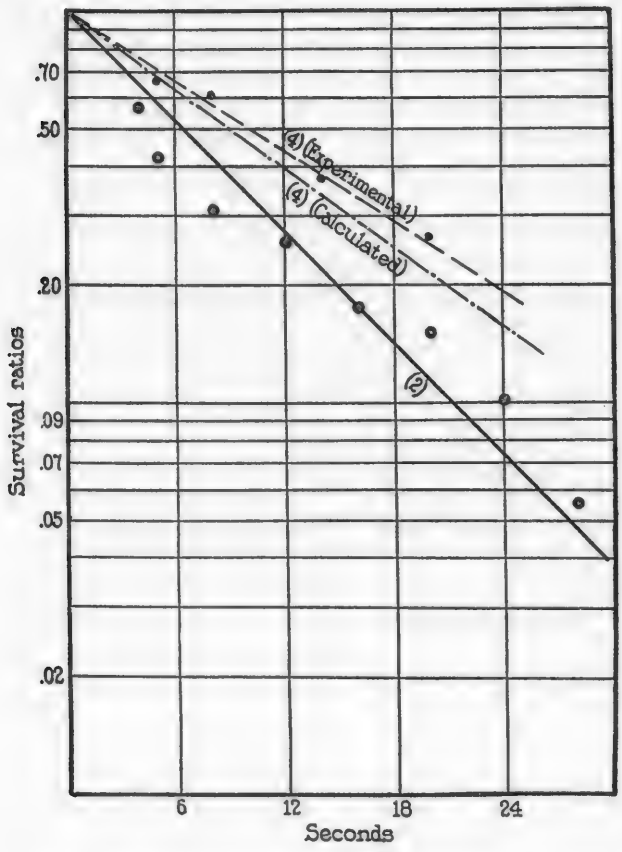
Results from Standardized Experiments

Experiment (Table I)	Electrons absorbed per bacterium per second	
	Observed	Calculated
(1) *B. coli*	ca 0.112	0.132
(3) *B. aert*	.056	.067
(4) " "	.068	.077
(5) *S. aureus*	ca .105	.114

of absorptions per bacterium per second as calculated and observed are listed in Table II. In each instance the theoretical number exceeds the experimental by about 15 per cent. On its face this would mean that only 85 per cent of the bacteria which absorbed electrons were killed, though whenever death was brought about, one electron was sufficient to accomplish it. In view of the inaccuracy of the measurements involved in standardization, however, too much importance cannot be attached to this percentage of killed organisms. It is perhaps safe merely to conclude that the number of bacteria killed is of the same order of magnitude as the number which absorb electrons.

The similarity in the curves for *B. coli* and *B. aertryke* might be expected from their similarity in size and shape. Though none of the experiments using *Staphylococcus aureus* gave results as uniform as with

the two bacilli, the observed greater resistance of this bacterium is adequately accounted for by its smaller size (Table II).



TEXT-FIG. 2. The data of this figure refer to Experiments (2) and (4) with *B. aertryke*.

CONCLUSIONS

1. For the two motile bacilli, *B. coli* and *B. aertryke*, the absorption of a single 155 K.V. electron is sufficient to cause death. Furthermore,

all, or nearly all, the electrons absorbed are lethal. The same is undoubtedly true of *Staphylococcus aureus*. In addition to providing a quantitative picture of the interaction of bacteria and cathode rays, these results suggest that radiation of the energy content used in our experiments is not suitable for altering the inheritable characteristics of bacteria.

2. The differences in sensitivity to cathode rays shown by the bacteria studied can be explained by the purely physical factor of size.

3. Counts giving significant conclusions concerning killing rates can be obtained only if there is no clumping of the cells when spread and only if the cells are not allowed to multiply before irradiation. Both these precautions seem rarely to have been met in the experiments that have in the past been made with X-rays and other forms of radiation.

EXPLANATION OF PLATE 30

FIG. 1. A photograph showing the anode end of the cathode ray tube and the apparatus used in irradiating the agar plates.

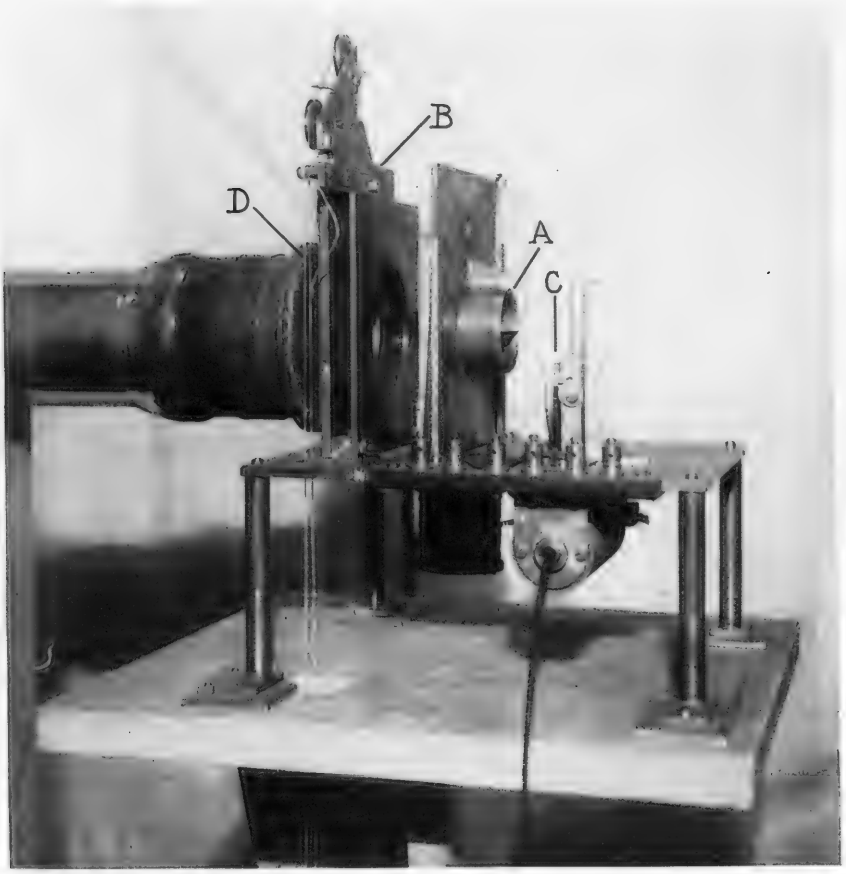


FIG. 1

(Wyckoff and Rivers. Effect of cathode rays upon bacteria)

RISE IN TEMPERATURE PRECEDING THE APPEARANCE OF SYMPTOMS IN EXPERIMENTAL POLIOMYELITIS*

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(Received for publication, March 31, 1930)

In the main the clinical manifestations and pathological lesions of experimental poliomyelitis in the monkey represent a faithful reproduction of the human disease.

The clinical course of the experimental disease is fairly uniform. The first symptoms vary somewhat in time of appearance. This depends largely on the strain of virus used, the extent to which the virus has been modified either by temperature (1), by chemical agencies (2), or by the addition of convalescent serum (3). When a 7- or 8-day unmodified virus is inoculated intracerebrally the monkey remains normal in appearance (after the effect of the operation and anaesthetic has worn off) until the seventh or eighth day. The first symptoms to appear are tremor of the head and the extremities (more easily noticed in the upper extremities), the ruffled coat and some awkwardness and stumbling when the monkey attempts to climb. Within a few hours these symptoms are followed by paralysis of varying severity and in most instances by complete prostration and death. When the disease does not terminate fatally the animal goes through a convalescent period of several weeks' duration, after which except for the residual paralysis it seems quite normal.

In one respect, however, the experimental disease had been considered as differing from the human form in that a phase of the disease analogous to the preparalytic stage of the human disease was lacking. As is pointed out in this paper this view is not justified. The preparalytic stage of the disease in human beings is now well established

* This work was supported by the Harvard Infantile Paralysis Commission, a fund privately donated to the Vermont Department of Public Health and a gift from the International Committee for the Study of Infantile Paralysis.

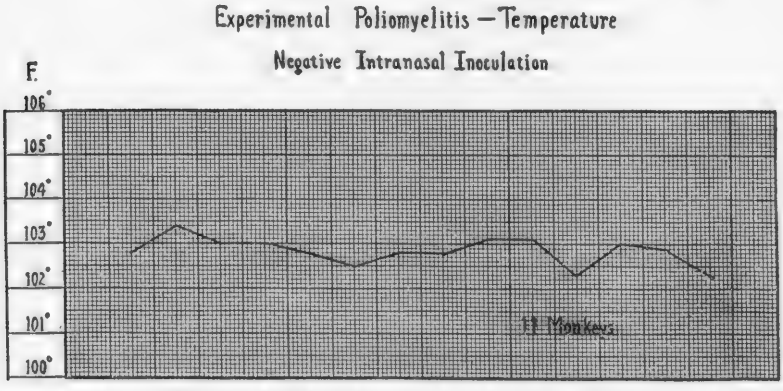
(4), lasting 2 or 3 days before the onset of paralysis and consisting of a syndrome of fever, headache, vomiting, stiff neck and back and a coarse tremor. These symptoms are regularly associated with an increase in the cell count and in the globulin of the spinal fluid. In the experimental disease paralysis follows the earliest symptoms so rapidly that a division comparable to the human preparalytic stage has not previously been often made. On account of the lack of an obviously preparalytic stage there is a question whether certain procedures applied to the inoculated monkey are applicable to the human disease in the preparalytic stage. This uncertainty has been felt particularly in experiments dealing with the effects of convalescent serum therapy on the course of the disease.

In an attempt to determine if there are some earlier detectable manifestations in the experimental disease comparable to the preparalytic stage of the human form, we undertook a series of studies on monkeys inoculated in the course of our work. These studies dealt with the temperature, formed blood elements, spinal fluid, and blood chemistry. Similar studies made on a group of normal monkeys served as controls.

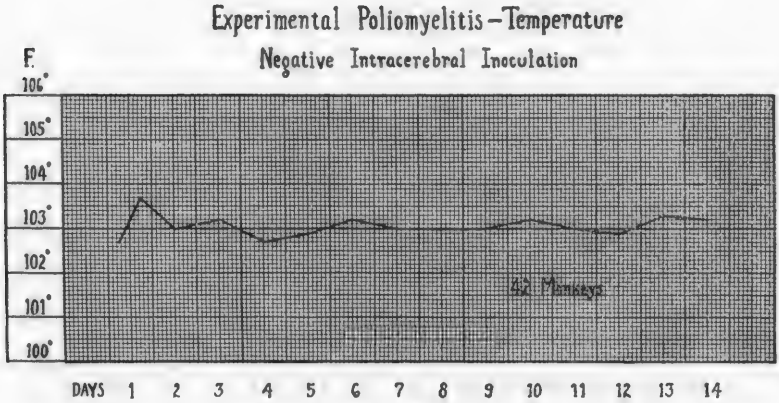
The present communication devotes itself to temperatures observed in normal monkeys and in monkeys infected with the virus of poliomyelitis. Rectal temperatures were used throughout. It was noted early that the administration of ether causes an immediate, though transient, drop in the temperature, so that temperatures were not taken at such times.

The temperature of the normal monkey is about 103°F., varying to some extent, however, without apparent reason in the same monkey both on the same day, and from day to day. Fluctuations of 1° and occasionally 1.5°F. between the maximum and minimum temperatures are not uncommon, but variations of more than 1° from the average mean temperature are rare. The average of 1301 readings on 46 monkeys over a period of several months was 102.9°F. A number of the monkeys showed a temperature consistently higher than 103°F., several ranging between 104° and 105°. The reason for these exceptions was not always clear, although some animals developed diarrhea, and in several instances where the monkeys came to autopsy a generalized tuberculosis was found. In others, however, there was no such explanation.

Graph I is a composite curve of 11 intranasally inoculated animals that resisted infection. The average temperature of 147 readings on



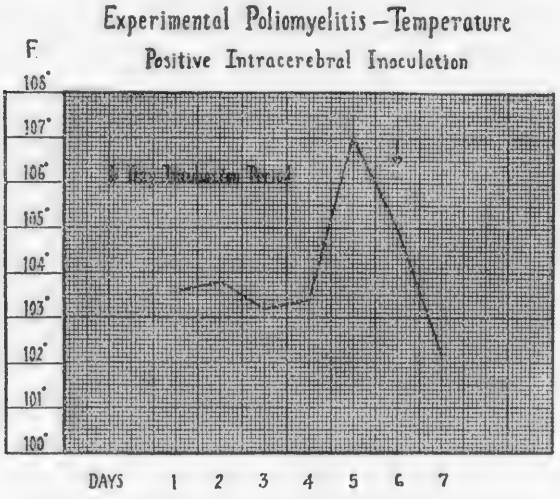
GRAPH I



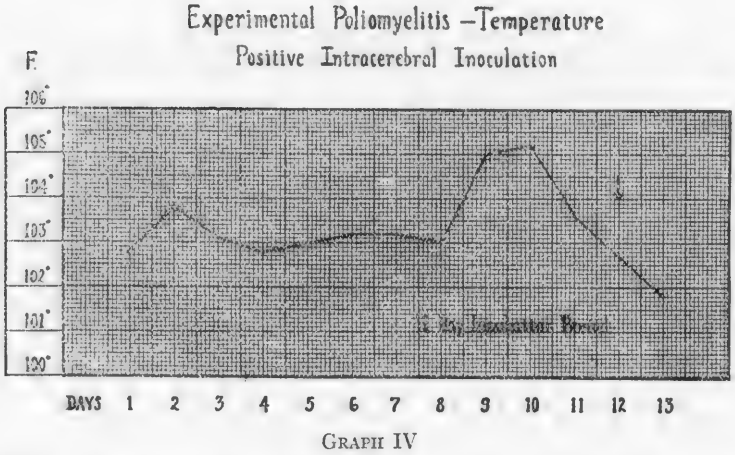
GRAPH II

these monkeys was 102.9°F., with a maximum plus deviation of 0.5°F., and a maximum minus deviation of 0.6°F. from the average.

Graph II is a similar composite curve of 42 intracerebrally inoculated animals that did not succumb to the disease. The average tempera-

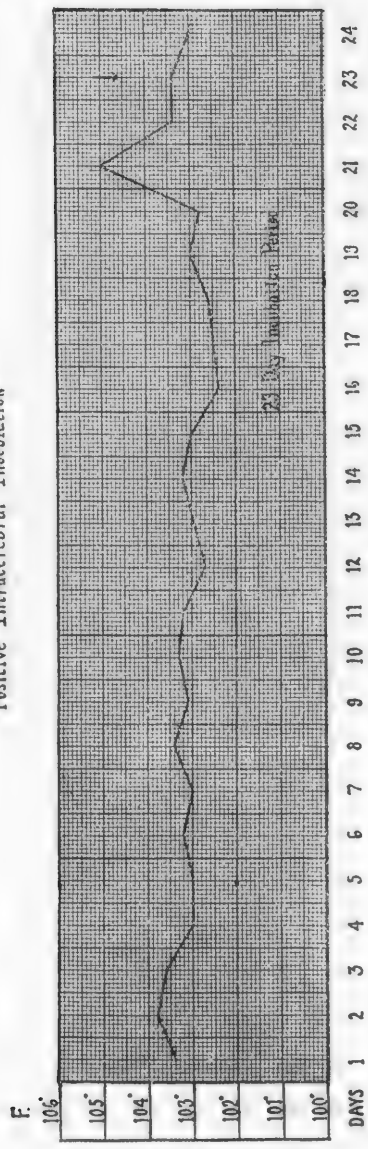


GRAPH III



GRAPH IV

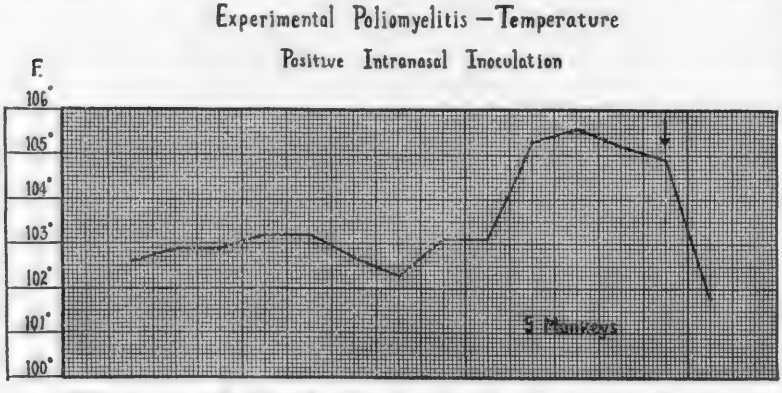
ture of 470 readings on these monkeys during the "normal" period was 103.1°. The maximum plus and minus deviations were 0.6° and


Experimental Poliomyelitis – Temperature
Positive Intracerebral Inoculation
F.
106°
105°
104°
103°
102°
101°
100°
DAYS 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
23 Day Incubation Period

GRAPH V

0.4°F. respectively. With the exception of the transient rise following intracerebral inoculation the two curves are identical. The preliminary rise of 1° to 2° following the operation was regularly observed in monkeys inoculated intracerebrally and usually did not last beyond the first 24 hours. This rise was absent in those monkeys in which the virus was instilled into the nostrils.

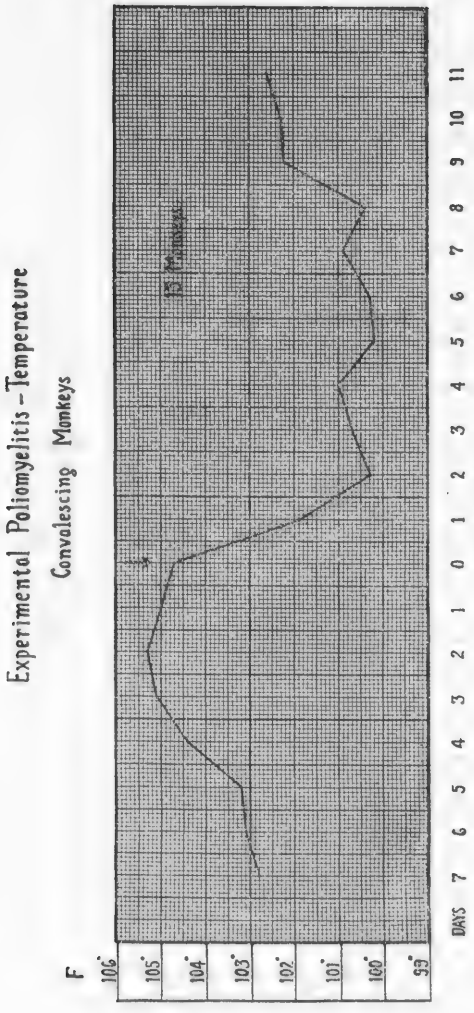
The temperatures of the monkeys which succumbed, however, showed constant and characteristic alterations from the normal. Graphs III,* IV, and V show the temperature curves of a number of intracerebrally inoculated animals that developed the disease. These



GRAPH VI

graphs show the preliminary rise, already referred to, followed by a varying period of "normal" temperature, followed in turn by an abrupt rise which reaches its maximum about 24 hours before the onset of the earliest symptoms. This interval of "normal" temperature has at times lasted 18 to 21 days, as in the instances of prolonged incubation period illustrated in Graph V. This type of curve occurred in about two-thirds of the animals that succumbed. In the remaining animals, however, the second rise was more gradual, although the temperature, as in the other animals, reached its maximum about 24 hours before the onset of symptoms.

* On this and subsequent charts the date of appearance of symptoms is indicated by an arrow.


Experimental Poliomyelitis - Temperature
Convalescing Monkeys
F
106°
105°
104°
103°
102°
101°
100°
99°
DAYS 7 6 5 4 3 2 1 0 1 2 3 4 5 6 7 8 9 10 11

GRAPH VII

Graph VI* is a composite temperature curve of 9 monkeys successfully inoculated intranasally. These animals did not show the early preliminary rise noted in Graphs III, IV, and V, but the temperature rise preceding the manifestation of symptoms appeared in a manner identical to that observed in the intracerebrally inoculated animals.

Coincident with this preparalytic rise in temperature, spinal fluid changes were observed similar to those in preparalytic human poliomyelitis. Details of these findings will be published in a later communication.

The trend of the temperature curve after the monkey is paralyzed and prostrated is illustrated in Graph VII.* With the appearance of the usual symptoms of the experimental disease the temperature is already declining. Within 24 hours after the appearance of paralysis it has usually become subnormal. When paralysis is extensive and the prostration marked the temperature has in several instances dropped as much as 10°F. That this pronounced drop in temperature is due in part to external factors is indicated by the fact that it was possible to avoid such an extreme drop by keeping the animal warm. The return of the temperature to the normal range in surviving monkeys seemed to depend considerably on the degree of prostration as well as the temperature of the environment. Several such monkeys were carefully nursed for a number of weeks and Graph VII shows the gradual return of the temperature to the normal range as the monkey's general condition improved.

SUMMARY

The data presented in this paper offer a means of earlier recognition of experimental poliomyelitis in the monkey. The early appearance of the temperature rise (1 to 3 days before the onset of the usual recognizable symptoms) associated with spinal fluid changes suggests that there is a stage in the experimental disease corresponding to preparalytic human poliomyelitis.

* Since some of these animals had been given repeated intranasal instillations of the virus, the incubation period was not ascertainable. Hence, the temperature readings are expressed in days preceding the onset of symptoms.

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ABNORMALITIES PRODUCED IN THE CENTRAL NERVOUS SYSTEM BY ELECTRICAL INJURIES*

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PLATES 31 TO 33

(Received for publication, February 27, 1930)

The problem of injuries following contact with electric circuits has become an important one owing to the extensive use of electricity in everyday life. In 1913, 670 deaths in the United States were attributed to this cause. Circuits supplying homes and factories are commonly of 110 or 220 volts, either alternating or continuous current. A 100 volt circuit may prove fatal. For this reason it has seemed important to make a careful fundamental study of electrical injuries with a view to saving the lives of those who have received a severe shock. The present paper presents experimental evidence concerning the injuries produced in the central nervous system.

The material serving as a basis for this study consists of some 286 rats, injured by either continuous or alternating electric circuits at different potentials. The experimental technique and the immediate results of the injury have been reported in another place (Langworthy and Kouwenhoven, 1930). The central nervous systems of these rats were preserved immediately after death and typical sections have been cut in an endeavor to correlate the experimental results with actual histological pictures. It will be possible to show that the abnormalities produced by the continuous current differ from those produced by the alternating.

Many fatal accidents from contact with electricity are attributed to the resulting fibrillation of the ventricles of the heart. Ventricular fibrillation is believed to be more common after contact with circuits

* This research was conducted with a fund provided by the Committee on Electric Shock.

at low voltages. Circuits at high voltages, on the other hand, are thought to cause death from respiratory failure due to a central inhibition in the nervous system. Since the present experiments were directed to an elucidation of injury in the brain and spinal cord, an experimental animal was chosen in which the heart recovers spontaneously from ventricular fibrillation. The rat proved to be such an experimental animal; it is doubtful if any deaths in this series can be attributed to cardiac failure. This makes it possible to focus attention upon the central nervous system.

The histological study reported here endeavors to demonstrate evidence of injury in the nerve cell itself as a result of electric shock. These changes are sought in the general appearance of the cell, in the Nissl granules and in the cell nucleus. These criteria of injury of the nerve cell have been used by many investigators.

A good review of the pathological changes produced in nerve cells by poisons, such as arsenic, phosphorus and veratrin, by anemia and by high temperatures was given by Barker (1909). It has been demonstrated that the Nissl granules are formed after death by the coagulation of some substance normally present in the cell. The substance is used in cell metabolism and is greatly decreased in amount by fatigue. As the result of injury, various changes are observed in the cytoplasm; among these may be suggested complete loss of granules, loss of granules at the periphery of the cell or around the nucleus and vacuolization. The cell may be swollen, the granules large and adherent in a compact mass or the cytoplasm stain a uniform blue. Later a contraction of the cytoplasm and pyknotic condition of the nucleus suggest cell death. Buzzard and Greenfield (1925) have more recently reviewed the subject of nerve cell pathology. Here it is important to discuss the changes that have been found in nerve cells following injuries with electric currents and closely allied types of traumata.

Urquhart (1927) felt that the asphyxia accompanying temporary respiratory inhibition was sufficient to produce changes in the cells of the brain and cord that would confuse any picture of injury produced by electricity.

Gomez and Pike (1909) previously found that anemia lasting 8 minutes was sufficient to kill the small pyramidal cells of the cortex. After 13 minutes there were marked changes in the Purkinje cells in the cerebellum. In the case of the medulla, anemia for 20 to 30 minutes produced alterations incompatible with complete recovery. Cannon and Burket (1913) later showed that cells of the myenteric plexus could recover from anemia lasting 6 hours. In the present experiments the periods of apnea were short; the rats that were saved breathed in almost every case within 2 minutes. It seems that the effects upon the nerve cells of anemia may be discounted in the present work.

Mott and Uno (1922) studied the central nervous system of patients that died from surgical shock from 24 to 48 hours after injury. The brains were fixed first in formalin and afterward blocks were placed in alcohol. In the cerebellum very few Purkinje cells showed a normal staining reaction. The Nissl granules were broken up into fine dust; the cell was swollen, the nucleus indistinct and the whole cell only faintly stained. In the neighborhood one could see cells which presented a more normal appearance.

Regarding the changes produced in the nerve cell by electrical injuries, observers do not agree. Small perivascular hemorrhages have been reported, especially in the medulla and floor of the fourth ventricle; these hemorrhages are common after legal electrocution. Chromatolysis of the ganglion cells and rupture of the cells with dislocation of the nuclei have been seen. Huber described a loosening of the glia, vacuolization of the nerve cells and approximation of the nuclei to the walls of the cells. Kawamura says that some cells appear normal while others are shrunken and hyaline. In the medulla oblongata the Nissl substance is broken into small granules. Mott and Schuster (1909) described the lesions in a man who recovered from a shock at 20,000 volt potential and lived for 7 hours thereafter. The autopsy was performed 39 hours after death.

Spitzka and Radasch (1912) sectioned the brains of five electrocuted criminals and found peculiar areas from 25 to 200 micra in diameter around small blood vessels. There was a central rarified zone and a peripheral condensed one. They explained their observations on the basis of a sudden liberation of gas bubbles by the electrolytic action of the current. Since the current used was alternating, it is more likely that the vacuolization resulted from excessive temperatures. In electrocution the brain becomes exceedingly warm, a temperature of 145°F. has been recorded.

MacMahon (1929) has performed experiments similar to those reported in the present paper. Guinea pigs were exposed to continuous circuits of low voltage. Some of the animals received a series of shocks on different days. Following the injury, excitability, nervousness and stupor were observed. There was also weakness, paralysis of the posterior extremities and diminished sensation. The animals were less active and did not move about quickly after the shock. Paralysis of both posterior extremities occurred in more than half the animals. In some cases it came on immediately after the shock; others did not become paralyzed until 2 days thereafter. When paralyzed, the animals dragged their hind feet and appeared to have no control of the sphincters. If the paralyzed legs were lightly stimulated by touch, pressure, pain or heat there was no response.

On examination of the central nervous system of the guinea pigs that had survived the shock it was found that the spinal cord was swollen, soft and almost diffluent. There were petechial hemorrhages in the pia arachnoid. The white matter of the cord was most seriously injured, and the lesion was greatest in the posterior columns. The earliest change in the nerve fiber consisted in a swelling of both the axis cylinder and myelin sheath. The myelin in later stages disappeared

leaving irregular vacuoles and spaces around and among the swollen axones. The peripheral nerves showed similar degeneration. Little information was given concerning changes in the nerve cells.

With this review of the literature, it is important to discuss the peculiar difficulties in interpretation of pathological changes in nerve cells as seen in sections stained with a basic dye. Criteria that are used by investigators as evidence of nerve cell damage have already been presented. But, in order that these criteria may be valid, other very definite postulates have to be filled.

To demonstrate the finer structure of cells, and in particular, nerve cells, it is extremely important that fixation of the tissue be carried out immediately after death. If the autopsy is delayed for only a few hours, the nerve cells stain a uniform, blue color with the basic dyes. Moreover the fixative is important; 95 per cent alcohol should be used if a sharp, clear staining of the granules in the cytoplasm is desired. Formalin is a standard fixative for nervous tissues but it permits only a poor differentiation of the granules. The 95 per cent alcohol has a very low power of penetration; with fairly large brains an arterial injection is important. The central nervous system of the rat is so small that the brain was not injected but was cut in thin slices and immersed in the fixative.

It is often difficult to obtain a good staining of the sections. A good thionin dye becomes slightly metachromatic and imparts to the granules a deep blue tint with a trace of lavender. The sections with this lavender hue do not fade easily and remain in good condition indefinitely. If the sections are overstained the cytoplasm of the cells is a uniform blue and the granules are not distinct; if the stain is light the granules do not receive their full value.

After a sharp staining of the cells, their nuclei and the granules in the cytoplasm is obtained, the interpretation still offers difficulties. Different groups of cells show great variation as to their size, shape and the arrangement of the granules. Characteristic patterns of the granules have been studied by Malone (1912 and 1913). He pointed out that somatic motor cells have large Nissl granules packing the cytoplasm of the cell. Autonomic cells, on the other hand, have large granules surrounding the edge of the cytoplasm and smaller granules in the remaining area. Sensory cells have fine, dust-like granules, scattered uniformly. The possibilities of granule arrangements in the higher centers of the brain are infinite.

To avoid difficulties of telling normal from injured cells by the arrangement of granules, only well known types of cells are discussed in the present report. The somatic motor cells of the anterior columns of the cord were examined in every case. It was most important to study the dorsal nucleus of the vagus because of its important func-

tion in respiration. The Purkinje cells of the cerebellum are characteristic as are the cells of the olives. Finally the sensory cells in the mesencephalic nucleus of the trigeminal were used as a type. No minute examination of the cells of the cerebral cortex was made. The sections of the forebrain were examined for hemorrhage and evidence of burning, as was the rest of the brain and cord.

Methods

The brain and spinal cord of the rats that died as an immediate result of the injury, were removed at once, cut into thin slices and placed in 95 per cent alcohol. After adequate fixation the blocks were imbedded in paraffin and sections stained with thionin. Rats that survived the shock were killed a few hours or days after the injury. In the case of a number of the rats, a microscopic examination of the thoracic and abdominal organs was made.

HISTOLOGICAL STUDY

The experiments are grouped on the basis of different potentials of alternating and continuous circuits. Greater injury was produced by the circuits at high voltages and these marked changes may be described first.

Rats Subjected to a Continuous Circuit at 1000 Volt Potential

It will be possible to demonstrate that the general microscopic picture of injuries produced with the continuous circuit is somewhat different from that produced by the alternating. Since the lesions are more severe and more clear cut with the 1000 volt circuit it is proposed to begin with this group.

Thirty-three rats were subjected to a continuous circuit at 1000 volt potentia for periods from quick manual closure and opening of the switch to 4 seconds. For the purpose of study a contact of ½ second was approximated as closely as possible. The current readings were 600 to 1100 milliamperes. Twenty of the rats died at once, nine recovered and four were paralyzed. These findings need further explanation; thus eight received the current for the time required to open and close the switch. Of these eight animals, five recovered, two died and one was paralyzed. Thirteen were shocked for approximately ½ second; four were normal, three paralyzed and six died at once. No rats survived an application of the current for 1 second or over.

By comparison it is found that the continuous 1000 volt circuit was more injurious to rats than the alternating circuit of the same potential.

This is particularly true if the time element is taken into consideration. But, at autopsy, few gross hemorrhages were found in the central nervous system, even of the paralyzed rats. Study of the sections gives an explanation for the fatalities in this group.

Twenty rats that died immediately will be considered first, in reference to the microscopic findings.

The photomicrographs of abnormal cells that illustrate this paper were made largely from sections of the central nervous system of this 1000 volt group. Large numbers of sections are available and the pathological picture is remarkably uniform. In three of these rats there was a large hemorrhage in the fourth ventricle that must have pressed upon the respiratory center. The brains, in general, were remarkably free from microscopic hemorrhages.

The surface of the cerebral cortex was not as severely injured as in the alternating circuit series. It is true that in several cases large cavities were found near the surface of the gray matter. These have been observed in the brains of electrocuted criminals and are supposed to be due to the collection of gases produced by the generation of heat. It is interesting that in the rats they are found most commonly close to the surface electrode where the concentration of electricity is great, although they do occur in all portions of the nervous system. They are often present in rats that recovered from the shock. In man they are perivascular; this is not necessarily true in the rats. The wall of these cysts is deeply staining due to the condensed brain tissue. In some of the rats the surface of the cortex appears to have actually burned and the remaining edge stains an intense blue.

The greatest interest centers around the changes in the nerve cells that are similar to those produced by the 1000 volt alternating circuit, but more severe. These may be described first in the somatic motor cells of the spinal cord.

The cells are selectively injured; some are grossly damaged and others appear almost normal. The cell in Fig. 4 from a rat of this series has a nucleus that appears practically normal. On one side of the cytoplasm the granules are numerous. On the other, toward the periphery of the cord, few granules are present. The cytoplasm appears shrunken and the outlines of the cell can be made out with difficulty. Since this cell has a normal nucleus it is thought that it has the potentialities of a complete recovery.

In Fig. 5 the cell presents a somewhat different picture. The nucleus stains deeply and the nucleolus is swollen but the nuclear injury is scarcely profound. Granules are numerous around the nucleus but the outer portion of the cytoplasm is relatively free from granules. This cell has suffered a loss of Nissl granules.

A severely injured cell is shown in Fig. 6; this opinion is given because of the

nucleus which is shrunken, and stains a uniform deep color in which no nucleolus or chromatin skeins can be seen. The clear area around the nucleus shows its normal size. The granules are numerous in this cell, although portions of the cytoplasm are quite free from them.

In Fig. 7 are two cells with nuclei slightly less damaged. The nucleous is greatly swollen. The remainder of the nucleus is shrunken and darkly staining although the color is not as deep as the nucleolus. In the upper cell the granules are sparse but scattered uniformly. The cytoplasm has no distinct boundaries in the lower cell and it would appear that the cell has ruptured and the granules are free in the perivascular space. It is doubtful whether these cells have the potential power of recovery.

The deep staining cell in Fig. 8 is similar to that in Fig. 6; the other two cells show severe damage. The nucleus in the lower one, however, appears quite normal. The cytoplasm of both these cells is greatly shrunken and the edges are jagged and irregular. If the nucleus is normal it is probable that the cytoplasm may be regenerated. Similar cells are abundant in all this material and suggest the severe injury responsible for the death of the rats.

The Purkinje cells are particularly vulnerable to injury with electric shocks.

The results of the shock, as seen in the rats that die immediately, are seen in Fig. 12. The injury is again selective although large groups of cells are abnormal as the photomicrograph suggests. The nuclei of the cells stain a uniform, deep color in which the chromatin and nucleolus cannot be distinguished. The cytoplasm likewise stains a lighter but uniform blue. That this injury betokens the death of the cell is clear from the examination of rats that have survived for some time (Fig. 11). Here the cells stain a dark color and are much smaller than normal.

Since sensory ganglia were not stained in every preparation, the cells of the mesencephalic nucleus of the trigeminal were used for illustrating damage to primary sensory neurones.

The nucleus of the cell (Fig. 16) is irregular in outline and small but the nucleolus may still be distinguished. The clear surrounding area was probably filled by the nucleus. The granules show poorly in the cytoplasm which stains a rather uniform blue. These cells are severely damaged. Fig. 15 shows several fairly normal cells and one with a deep staining, pyknotic nucleus. Sensory cells are relatively resistant to injuries by electricity.

It was considered particularly important, in each case, to examine the dorsal nucleus of the vagus since the cells of this nucleus have an important part in initiating and controlling respiration.

These autonomic cells normally have large granules at the periphery of the cytoplasm and smaller granules scattered through the remainder. A fairly typical illustration of the granules is seen in Fig. 17. However, numbers of the cells in these rats, as shown in Fig. 17, have deep staining nuclei suggesting the death of the cells. One of the cells in Fig. 18 has a deep staining nucleus; in another the edges of the cytoplasm are indistinct and a loss of granules has occurred. More severe injury is seen in Fig. 22 where both nuclei and cytoplasm are grossly injured. If many cells of the nucleus are thus damaged not a temporary block but a permanent paralysis of the respiratory center is to be expected.

Four rats were paralyzed following the shock. There was a tendency toward recovery so that the legs became much stronger in one of the preparations and by the fourth day they were used fairly well. Nocuous stimuli applied to the paralyzed portion of the body elicited a response in all of these rats, in contrast with the alternating group. Two of them became incontinent on the second day, although no blood was ever present in the urine. All the alternating group were incontinent and the urine was deeply stained with blood. Moreover, priapism was present in these rats; this suggested an irritative lesion in the spinal cord. Macroscopic examination of the brain and spinal cord revealed no lesions that would account for the marked symptoms.

These rats lived from 2 to 6 days following the shock. On microscopic examination of the cord it was found that many ventral horn cells had recovered from the initial injury and were fairly normal in appearance. The granules, however, were not well defined and the cytoplasm tended to stain a uniform deep blue. Other groups of cells were present which showed practically no granules in the cytoplasm. The third type of injury, found particularly in the lumbar portion of the spinal cord was the most serious and characteristic; it is represented in Fig. 9. The cells are deeply staining and the cytoplasm has irregular contours. The nucleus is distinguished with difficulty; in the upper cells and the one on the lower right, the nucleolus may be recognized, staining a little deeper color than the cytoplasm. The cytoplasm is very shrunken. The two lower cells are being attacked by phagocytes that have penetrated the cytoplasm of the cell. That this is not the nucleus is clear in the lower right hand cell where both the nucleolus of the nerve cell and the phagocytic cell may be distinguished. This phagocytosis is evidence of severe cell injury and death. We have, then, in this group, paralysis of the posterior

portion of the body, not due secondarily to the influence of a large hemorrhage upon the cells and fibers of the cord but to an actual injury of the nerve cells. This injury is not as widespread as in the cases of hemorrhage and only groups of nerve cells are injured. It is important to speculate as to whether the hemorrhage was the sole factor producing the paralysis in the alternating circuit series. Since the tracts were not severely injured in the present group sensation was not abnormal. Irritation of the autonomic cells of the cord produced the priapism and severe damage of similar cells was responsible for the incontinence. The atony of the bladder musculature was not great enough to produce hemorrhages and subsequent hematuria. As the cells tended, to some extent, to recover their functions, the abnormalities became, in some cases, less marked.

Nine of the rats recovered and lived for periods from 5 hours to 3 days. There is considerable infiltration of the ventral columns of the cord with phagocytic cells. The motor cells, in general, stain deeply 5 hours after the injury but the Nissl granules are poorly defined even after a period of days. Many cells are necrotic.

The injury of the Purkinje cells in the group that survived may be seen in Fig. 11. The severely injured cells become shrunken and pyknotic, forming a conspicuous group, contrasting sharply with the normal cells. Others (Fig. 13) not as severely injured show large vacuoles in the cytoplasm Fig. 14 again demonstrates the dead cells. Large numbers of Purkinje cells are injured and the damage is greatest on the dorsal surface of the cerebellum.

Fig. 21 is a photograph of the dorsal nucleus of the vagus from the brain of a rat surviving the shock for 4 days. The shrunken, pyknotic, dead cells are sharply contrasted with the normal autonomic cells. A somewhat different pathological picture in the dorsal nucleus of the vagus is given in Fig. 20. At the lower edge is a fairly normal cell and at the upper edge a shrunken pyknotic one. Between are two cells that have completely disintegrated.

Fig. 19 shows the injury as observed in the superior olive of a rat that survived the injury. Contrasted with normal cells are others with deeply staining cytoplasm and nucleus.

Rats Subjected to Continuous Circuits at 500, 220 and 110 Volt Potential

The histological findings in groups subjected to these voltages will be summarized together. Only one type of abnormality was observed as an immediate result of the injury that has not been mentioned in

connection with the 1000 volt circuit group. Five rats subjected to a continuous circuit at 500 volts exhibited typical clonic convulsive seizures, some 5 or 10 minutes after the shock. These convulsive attacks occurred spontaneously or upon touching the rats, which were definitely hyperirritable. They never continued longer than an hour and the rats were perfectly normal thereafter. No gross lesions were demonstrable in the central nervous system. No histological change was found to explain the convulsions; it is true that these animals lived for some time after the injury and after the convulsions had ceased. The outer portion of the cytoplasm of many of the ventral horn cells was relatively free from granules. No lesion was found in the vestibular nuclei.

The cellular injury in the rats that died immediately was much less severe after shocks with the lower voltages. In like manner, recovery of the abnormal cells was more rapid. Some trauma to the Purkinje cells was observed with the longer applications of the current. There was in every case a definite diminution of the Nissl granules. The impression is gained that prolonged artificial ventilation of the lungs might have saved many of these animals.

Rats Subjected to an Alternating Circuit at 1000 Volt Potential

Twenty-eight rats were exposed to an alternating circuit at 1000 volt potential; only one received the current for 4 seconds and the remainder for 1 to 3 seconds. The current produced a severe injury at the site of the electrodes; in the case of the head electrode there was often a burning of the brain. Of the 28 rates, 11 were paralyzed; 9 died at once and 8 were normal.

At autopsy ten of the eleven paralyzed rats showed a typical hemorrhagic lesion of the spinal cord that could be recognized grossly. They all died within 3 days after the injury. Sections showed a similar hemorrhage in the eleventh so that it may be assumed that paralysis in each case was due to a hemorrhage into the spinal cord.

These hemorrhages occurred frequently in the rats shocked with alternating circuits at all voltages. The bleeding occurred most commonly in the lower thoracic region, the hemorrhage extending in both a cranial and caudal direction. It was most commonly produced by the rupture of the posterior spinal artery and from this point the blood spread out into the posterior columns (Fig. 1). Several

rats that appeared normal during life proved to have a localized injury of this type. With further extension, the blood broke into the central canal of the cord which permitted a greater spread. The anterior horn cells and nerve fibers of the ventro-lateral columns were frequently pressed upon and paralysis ensued. In many cases the spinal cord was necrotic in the thoracic region (Fig. 2), indicating a complete transverse lesion. It is interesting that a spinal ganglion beside a completely necrotic area of spinal cord showed little change in the cells. The cytoplasm of these cells was pale and contained few granules.

The hemorrhage did not always involve the posterior column fibers first; other hemorrhages were observed in the ventro-lateral columns. It was rather common to find blood bilaterally in the ventral columns due to the rupture of the small arteries that travel with the ventral roots. The sensory pathways were injured so that nocuous stimuli applied to the posterior portion of the body elicited no response. The rats were in every case incontinent and, after a few hours, large amounts of blood appeared in the urine.

At autopsy it was found that the bladder was filled with blood clot. The atonic bladder had become so enormously distended with urine that hemorrhages had occurred in the wall and blood escaped into the urine. The paralyzed rats were never active and never ate following the shock. They could only be kept alive for relatively short periods.

The mechanical pressure of the blood had a severe effect upon the motor cells of the cord; this is shown in Fig. 3. The granules in the cells are very sparse. In the largest cell there are a few granules around the nucleus but the outer portion of the cytoplasm is quite free from them. The nucleus of the large cell is displaced toward one edge of the cytoplasm.

Concerning the mechanism of the hemorrhage, one possible theory is this. During the passage of the current an extremely powerful contraction of all the musculature of the body occurs. This is strong enough to tear the muscle fibers and frequent hemorrhages are found, for example, in the deep back musculature. The sudden flexion of the back that occurs when the current is applied may be sufficient to cause a rupture of small arteries in the cord. With a continuous circuit the contractions of the musculature are not as strong and the hemorrhages in the nervous system are few. The hemorrhage may, on the other hand, be attributed to venous stasis during the shock and the high blood pressure that immediately follows the injury.

Developing hemorrhages in the spinal cord were found in four of the rats that died as an immediate result of the shock. Likewise small hemorrhages, involving the posterior columns of the cord only, were present in two of the rats that appeared perfectly normal after the injury.

In all of the rats subjected to the alternating circuit at this potential, there was some injury of the dorsal surface of the brain, due to burning in the region of the head electrode.

This injury was severe over the cerebral cortex. There was a superficial loss of gray matter and the remaining edge was left as a deeply staining seared area. In several of the rats that survived an abscess occurred in this area. Large circular holes occurred just beneath the surface of the cortex without relation to the blood vessels. These are thought to be produced by the great generation of heat. The surface of the cerebellar cortex was, in many cases, injured in a similar manner and the cell injury was greatest close to the surface. In the brain stem there were clear, unstained areas surrounding the small blood vessels. These vacuolated areas have been described in the brains of electrocuted criminals.

The changes in the nerve cells may be described first as they were seen in the nine rats that died as an immediate result of the shock.

The abnormalities are marked and increase in severity in direct ratio with the time that the current is allowed to flow. After a 3 second application the granules in the ventral horn cells are greatly decreased in number. Fig. 4 may be taken as an example of such a cell although the animal was injured with a continuous circuit. The cytoplasm is shrunken and the irregular outlines of the cell are enclosed by a dilated perivascular space. Often it appears that the cell membrane has ruptured and the granules escaped.

The histological appearance of a group of cells, for example the ventral horn cells, is not uniform in a section. While the previous description holds for many of them, others appear shrunken and more deeply stained than normal. In these deep staining cells the granules can no longer be seen and the whole cytoplasm takes the deep blue color. Others cells appear almost normal in appearance. It is clear, therefore, that the nerve cells of the same group are not injured to an equal amount by the electric current; some are killed, some are injured and others are practically normal.

Perhaps the most important information concerning the extent of the cell injury is obtained from an examination of the cell nucleus. This has been illustrated by the photomicrographs from sections of rats injured with the continuous currents.

Thus the cell in Fig. 4 has a relatively normal nucleus with the chromatin arranged in finer skeins and the nucleolus sharply defined. In two cells in Fig. 3, normal nuclei are seen. They appear in the cells as relatively clear areas in a cytoplasm packed with deeply staining granules. In Fig. 7, showing cells severely injured by the current, the nuclei have a different appearance. The whole nucleus

is shrunken and takes a uniform bluish stain with scattered, deeper staining chromatin strands. It contains a swollen nucleolus. In the lower of the two cells in the photograph, the edges of the nucleus are irregular in outline.

The nerve cell in Fig. 6 is even more severely injured. The nucleus is greatly shrunken and is surrounded by a white, unstained area which is free from granules. The whole nucleus stains a uniform bluish color in which chromatin and nucleolus cannot be distinguished. A similar condition is seen in the darkly staining cell in Fig. 8.

From a study of this material, it has been concluded that a prognosis concerning the future recovery or death of a cell can be given from examination of the nucleus. If the nucleus appears normal, it is to be expected that the cell will recover its normal structure and function, no matter how severe the injury of the cytoplasm and granules. Thus, it is thought that the damaged cell in the lower portion of Fig. 8 has a good chance of recovery. On the other hand, a uniform staining, dark, shrunken nucleus betokens a cell that has no potentialities for recovery. Further support for this theory will be given in later pages of this report.

It has already been suggested that histological examination of the nerve cells from the central nervous system of rats that died as an immediate result of the shock shows that not all the cells were equally damaged. A whole range of variation may be observed in one single group. This variation may be due in some way to differences in the chemical composition or relative fatigue of these different cells. It may well be that death or functional damage following the shock is proportionate to the number of cells severely injured.

Changes similar to those described for the somatic motor cells in the cord are found in the brain stem and in particular, in the dorsal nucleus of the vagus. A slight damage of cell groups in the medulla might account for temporary respiratory block; more severe injury means the death of the rat.

The Purkinje cells of the cerebellum are easily injured by toxic poisons, by anemia and similarly by the electric currents. In sections from normal animals, it is frequently observed that a few of these cells are shrunken and pyknotic. The injury is so much more extensive in the present series that there can be no doubt as to its pathological significance.

These changes in the Purkinje cells are more marked in the rats that survived the shock by hours and days and less clearly demonstrable in those that died following the injury. Fig. 10 shows normal Purkinje cells and the light staining, large cells in Fig. 11 are even more characteristic. The cytoplasm contains fine scattered granules that give a foam-like appearance to the cell. In the rats that died, following the shock, the nucleus tends to stain a rather uniform blue color in which the chromatin and nucleolus can still be distinguished (Fig. 12). The granules in the cytoplasm are indistinct and the whole cell stains a light blue.

The animals that recovered were allowed to live for various periods of time; signs of recovery of the cells are clearly seen in the sections.

One rat died at the end of 6 hours. The number of cells with blue staining nuclei suggests that the cell damage had been profound. Even at this time the picture is different from that immediately following the shock. Certain cells have acquired a deeper staining of the cytoplasm, although it does not appear as distinct granules but, rather, as a uniform blue color. This is most clearly seen in the Purkinje cells. The latter are always more severely injured close to the dorsal surface of the cerebellum where the concentration of current was doubtless greatest.

Rats that have survived 2 and 4 days show further repair of the nerve cells.

The anterior horn cells at the end of 2 days are still shrunken and surrounded by a large perivascular space. The cytoplasm stains deeply and the granules are beginning to stand out clearly. Many of the nuclei have a bluish tint and the nucleolus is swollen. Other cells have made no recovery and appear to be disintegrating. After 4 days the cells are not yet completely normal in the structure and arrangement of granules in the cytoplasm.

Repair seems to be slower in the brain stem so that even at the end of 4 days many cells in the dorsal vagus nucleus contain few granules in a cytoplasm that is distinctly vacuolated. Here, deeply staining, dead cells may also be seen.

The picture in the Purkinje cells of the cerebellum is very characteristic at this stage and is represented in Fig. 11. Numbers of the cells, scattered among those of normal appearance, are shrunken and pyknotic, staining an intense color. These cells are found everywhere but are more common near the dorsal surface of the cerebellum. Where many of them are dead there appears to be a great disintegration of nerve fibers so that the Purkinje cell layer is easily pulled away from the granular layer, leaving a marked fissure in the sections.

The histological picture of the nerve cells in the paralyzed rats corresponds closely in general to that described for those that appeared normal when killed.

Due to the hemorrhage and subsequent degeneration of nerve fibers, the cord and brain stem were found to be markedly soft when removed at autopsy. In the completely necrotic portions of the cord the nerve cells are of course completely disintegrated; in other areas they are compressed by the hemorrhage producing characteristic changes. The nucleus is displaced to the periphery of the cytoplasm and there is a distinct loss of granules particularly in the periphery of the cytoplasm. In the areas of hemorrhage numbers of white blood cells are found, a majority of them polymorphonuclear. They wander into the gray matter around the injured cells.

The nerve cells in the brain stem make a slow and incomplete recovery in the paralyzed rats. After 2 days, the length of life of most of this series, the dorsal vagus cells still show an injury almost comparable to that found immediately following the shock.

With these marked changes found in the nerve cells it does not seem strange that numbers of the rats never breathed following the shock and that artificial respiration had to be used in the case of the others. The inactivity for the first 24 hours in those that recovered completely and throughout their lives in the paralyzed rats may be explained on the same basis.

Rats Subjected to Alternating Circuits at 500, 220 and 110 Volt Potentials and at 1000 and 500 Volt Potentials with the Voltage Not Maintained

It is scarcely justifiable to discuss the histological picture found in the central nervous systems of each of these groups of rats separately The relative mortality and paralysis produced at those different potentials have been thoroughly summarized in a previous paper. It is sufficient to point out that the number of rats killed or paralyzed became smaller, as the voltage and the amperage of the circuit were decreased. The shock could be made lethal, however, in every case by increasing the time of current contact.

The changes found in the nervous system were similar to those already described.

Actual burning of the brain occurred only with circuits at 1000 and 500 volts potential. Hemorrhages in the spinal cord were demonstrated in all the rats that were paralyzed following the shock. Hemorrhages were common in the groups in which the potential of 1000 or 500 volts fell considerably, immediately following the initial shock. It must be assumed, therefore, that the initial shock

at high voltage is very important in the development of this lesion. A section from the spinal cord of one of these rats is reproduced in Fig. 1. The conclusion was reached that hemorrhages are seldom responsible for the immediate death of the animal. Except in those cases where a hemorrhage occurs in the fourth ventricle, the respiratory block or paralysis must be due not to hemorrhage but directly to cell damage. This will be clearer when the group of rats shocked with the continuous circuit has been discussed.

As regards the cell changes, they were quite marked in the 500 volt series but decreased markedly in the 220 and 110 volt groups. They could be demonstrated, however, in each group by lengthening the duration of the contact. Recovery of the cells in the rats that survived was more rapid at the low voltages. More of the cells showed the potentialities of recovery. In the groups subjected to the lower voltages, it is probable that death was due, in most cases, to a temporary block in the respiratory center.

DISCUSSION

The injuries produced by the alternating circuits were somewhat different from those produced by the continuous. Hemorrhages in the central nervous system were common after contact with alternating circuits; the damage to the nerve cells was more severe after injury with the continuous circuits.

The hemorrhages following the alternating current shocks occurred not only in the nervous system but in other portions of the body. Blood vessels were found to have ruptured in the mucous membranes and autopsy study showed hemorrhages in the striated muscles. Blood escaped into the subarachnoid space, into the ventricles of the brain and the central canal of the spinal cord. Hemorrhages in the fourth ventricle were responsible in some cases for respiratory paralysis. Other characteristic hemorrhages occurred in the substance of the brain and spinal cord. In the latter position they caused in some cases a complete transverse necrosis and were responsible for the paralysis of the posterior portion of the body. As a result of the cord injury, hemorrhages occurred in the wall of the atonic and distended bladder and blood appeared in the urine. In the cord the pressure of the blood produced characteristic histological changes in the ventral horn cells.

The mechanism of these hemorrhages after injury with alternating circuits is not entirely clear. It has been suggested that it is due to the strong tetanic contraction of the back musculature at the time

the circuit is closed. It might on the other hand be attributed to the marked venous congestion that pertains during the shock or the sharp rise in blood pressure immediately following the injury.

Developing hemorrhages in the central nervous system were found in many of the rats that died at once and small hemorrhages in many that appeared to recover completely. If the hemorrhages involved a large area in the brain stem or cord the animal obviously had no hope of complete recovery. These hemorrhages were common with all voltages although they were more frequent with the higher potentials. They were obviously a large factor in the immediate or subsequent death of rats subjected to the alternating circuits.

With the alternating circuits at the higher potentials of 1000 and 500 volts there was injury of the dorsal surface of the cerebral and to a less extent of the cerebellar cortex. These regions were close to the head electrode where the density of the current must have been great. Indeed injuries of the subcutaneous tissue and bone were evident at autopsy. The surface of the cortex was seared and the edge stained an intense blue. Often large cavities were observed in the gray matter of the cortex and, less commonly, scattered throughout the central nervous system. They are supposed to be due to the collection of gases produced by the intense heat of the injury. They were found in rats that appeared normal after the injury. The cavities were surrounded by a deeply staining, condensed area of brain substance. They were not necessarily perivascular. If the rats survived the shock for several days, an abscess occurred on the surface of the cortex in a number of cases. This is interesting in that marked cellular reaction is believed to be retarded following electrical injuries.

The nerve cells showed marked abnormalities particularly with circuits at 500 and 1000 volts. After study of the sections it was believed that an accurate prognosis could be given concerning the death or subsequent recovery of the cells. If the nucleus was shrunken and stained an intense deep color so that chromatin skeins and nucleolus could not be distinguished, the cell was dead. In slightly less severe injuries the nucleolus was swollen and the whole nucleus was shrunken and stained darkly. A cell with a normal clear nucleus has the potentialities of recovery. In many cases the Nissl granules were greatly decreased in number and the outlines of the cells shrunken and irregular.

The cellular injury appeared to be selective so that a few abnormal cells would be found among a group comparatively normal. The Purkinje cells seemed to be most markedly injured and changes in these cells were found even after contact with circuits at 110 and 220 volts. Likewise injured cells were observed in the dorsal nucleus of the vagus, among the somatic motor cells of the cord and in the olives. The primary sensory neurones are resistant to injury.

In the animals that recovered, the cytoplasm of the cells became rather shrunken and stained deeply within a few hours but it was several days before the granules looked normal and distinct. The dead cells became more shrunken and pyknotic and finally disintegrated.

The cellular damage was even more marked in the rats subjected to continuous circuits at 1000 and 500 volts potential. This explains the fact that at 500 volts the continuous circuit was as dangerous as the alternating for the rats; at 1000 volts it was far more dangerous. Almost no hemorrhages were found after the continuous circuit shocks. The changes in the nerve cells have been adequately illustrated in the photomicrographs.

A small number of rats were paralyzed after contact with the continuous circuit but the picture was not the same as with the alternating circuits. There was a tendency to gradual recovery. Incontinence was not as marked a feature and blood was never present in the urine. Moreover there was no sensory loss in the paralyzed area. Microscopic examination showed that these paralyses were due, not to hemorrhage, but to the injury or death of groups of cells in the cord. These cells appeared shrunken and both the nucleus and cytoplasm stained a deep color. An infiltration of the anterior horns was present and phagocytosis of these dead cells observed. Convulsions and priapism occurred in this series and were probably due to the irritation of cells in the nervous system.

The alternating and continuous circuits at 220 and 110 volts potential produced much less injury to the nerve cells. Hemorrhages were common with the alternating circuit. It seems possible that a respiratory block was produced in these groups which might be relieved with adequate, long continued, artificial respiration. It is true, however, that long continued contacts with these circuits greatly increased the damage in the nervous system.

It is believed that definite abnormalities have been demonstrated in the nervous system that account for the death and abnormalities produced in the rats. With the circuits at lower potential these changes are not as marked and it is quite probable that temporary respiratory block was responsible for death. When a large hemorrhage occurred in the brain or cord or where a large number of nerve cells were permanently damaged, recovery could not be expected.

The fact that nerve cell changes may be demonstrated in animals dying at once following the electric shock indicates the explosive suddenness with which these abnormalities are produced. It is almost as if the cell had been blown to pieces by a charge of dynamite. The electric current must react violently upon the electrolytes producing profound dislocations of the nucleus and cytoplasmic material. While most other pathological changes in cells develop slowly this powerful physical agent produces abnormalities that may be demonstrated at once by means of even the rather gross methods of histological study available at the present time.

SUMMARY

The alternating and continuous circuits produced different types of lesions in the central nervous system. Hemorrhages were common after alternating current shocks and few hemorrhages were observed in the continuous circuit group. With both types of circuits at 1000 and 500 volts potential, severe abnormalities in the nerve cells were observed. These were more marked in the continuous circuit group. A uniformly staining, shrunken, pyknotic nucleus was taken as a criterion of nerve cell death. The Purkinje cells of the cerebellum were most susceptible to the current. Injured cells were studied in the dorsal nucleus of the vagus, in the somatic motor group, among the primary sensory neurones and in the olives. Changes in the histological structure of the cells in reference to recovery have been discussed.

Injury to the cerebral and cerebellar cortices occurred on the dorsal surface close to the head electrode. Small cavities were produced, particularly in the cerebral cortex, as the result of the circuit contact.

With the continuous and alternating circuits at 110 and 220 volts potential less severe changes were observed in the nerve cells although

hemorrhages were common in the alternating circuit group. It must be assumed in these cases that death was due to respiratory block rather than actual death of the cells.

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EXPLANATION OF PLATES

PLATE 31

FIG. 1. The rat was subjected to an alternating circuit at 1000 volts for 1 second. A large hemorrhage is present in the posterior columns of the cord. The rat survived the shock and behaved normally until killed 4 days later.

FIG. 2. There is a complete transverse necrosis of the cord following an extensive hemorrhage. The nerve roots appear fairly normal. The posterior portion of the body was paralyzed and there was no sensory response from the paralyzed area. Incontinence followed the shock and large quantities of blood were present in the urine. The animal was inactive and did not eat. The injury followed contact with an alternating circuit at 1000 volts potential for 3 seconds.

FIG. 3. Somatic motor cells of the anterior horn of the spinal cord which have been subjected to pressure from a hemorrhage. The nuclei appear normal except that one occupies an eccentric position. There are few granules in these cells. In the largest cell the granules are more abundant around the nucleus and the peripheral cytoplasm is clear and unstained. This rat was subjected to an alternating circuit at 1000 volts for 1 second.

FIG. 4. This is a somatic motor cell from the anterior gray matter of the spinal cord. The nucleus is normal. The cytoplasm on one side stains deeply and contains many granules; on the other side toward the periphery of the cord the granules are few and the cytoplasm is shrunken and irregular. The rat was shocked with a continuous circuit at 1000 volts for the time required to close and open the switch manually and died at once.

FIG. 5. The nucleus of this primary motor neurone of the cord is normal. Granules are present around the nucleus but the outer cytoplasm is relatively free from them. The rat was injured with a continuous circuit at 1000 volts for ½ second and died at once.

FIG. 6. This somatic motor cell of the cord must be considered damaged beyond hope of recovery. The nucleus is shrunken and stains a uniform black so that the nucleolus and chromatin skeins cannot be distinguished. It is surrounded by a clear area indicating the normal size of the nucleus. The granules in the cytoplasm look fairly normal. The rat died at once following contact with a continuous circuit at 1000 volts for 1 second.

FIG. 7. The nuclei of these two cells are shrunken and the outlines are irregular. The nucleoli are swollen although they can still be distinguished. They represent an intermediate amount of nuclear damage between Figs. 5 and 6. These anterior horn cells have many granules in the cytoplasm. In the lower cell the cytoplasm is shrunken; the outlines of the cell are irregular and indistinct. The rat was injured by a continuous circuit at 1000 volts for ½ second and died at once.

FIG. 8. The deep staining cell resembles that in Fig. 6. The other two anterior horn cells have shrunken, vacuolated and irregular cytoplasm with few granules. The lower cell however has a normal nucleus. The rat was subjected to a continuous, 1000 volt circuit and died at once.

FIG. 9. These are greatly shrunken, pyknotic anterior horn cells. The rat was shocked with a 1000 volt continuous circuit. Following the injury the posterior portion of the body was paralyzed. At the end of 6 days the rat was killed. Nucleoli can be observed in the cytoplasm of the two cells on the right. Phagocytic cells have eaten into the cytoplasm of the two lower nerve cells.

PLATE 32

FIG. 10. These are normal Purkinje cells. The cytoplasm contains delicate flaky granules.

FIG. 11. Normal Purkinje cells are seen in the upper portion of the photograph. The lower cells are shrunken and pyknotic and no distinction between nucleus

and cytoplasm can be made out. This rat survived a contact with a 1000 volt continuous circuit for 4 days.

FIG. 12. All the Purkinje cells in the photomicrograph are damaged. The nuclei are shrunken and stain a uniform black. The cytoplasm also stains deeply. These cells show the immediate effect of the injury since the rat died at once after a 1 second exposure to a 1000 volt continuous circuit.

FIG. 13. These Purkinje cells show a different type of injury in that the cytoplasm is greatly vacuolated, particularly at the edge next to the granular layer. The rat survived 4 days after contact with a continuous 1000 volt circuit.

FIG. 14. This picture again shows shrunken dead Purkinje cells from the central nervous system of a rat that survived 6 days after contact with a 1000 volt continuous circuit.

FIG. 15. These cells are from the mesencephalic nucleus of the trigeminal and indicate changes in primary sensory neurones. Sensory cells are relatively resistant to electrical injuries. One cell however has a shrunken pyknotic nucleus. The rat died at once after shock from a 1000 volt, continuous circuit for 1 second.

FIG. 16. Two cells of the mesencephalic trigeminal nucleus have injured nuclei. The nuclei are surrounded by clear areas indicating their normal size. The granules in the cytoplasm are not sharply discrete. Again the rat was subjected to a 1000 volt continuous circuit and died at once.

PLATE 33

FIG. 17. These are cells from the dorsal nucleus of the vagus. The arrangement of the granules is fairly normal for autonomic cells and the cytoplasm appears to have suffered little damage. The nuclei however stain a uniform dark color and it is thought the cells have been killed. The rat died at once following contact with a continuous, 1000 volt circuit for 1 second.

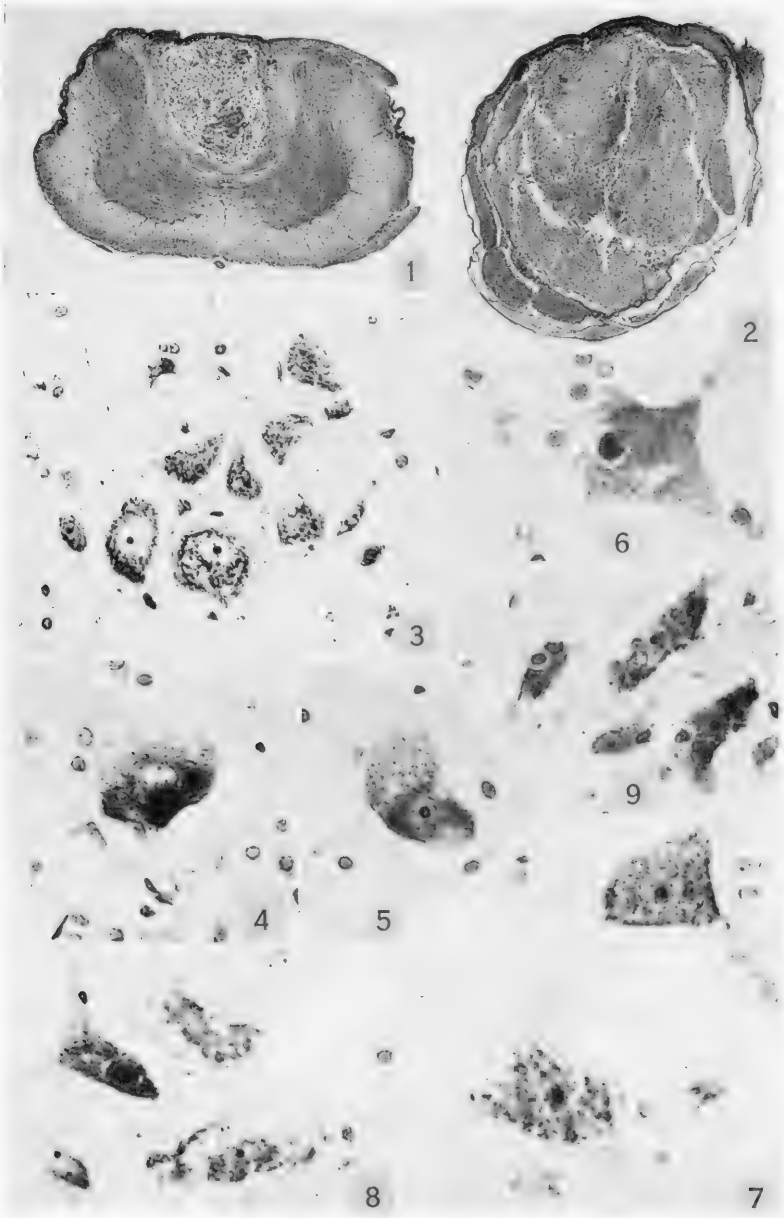
FIG. 18. The dorsal nucleus of the vagus. The upper cells appear normal; the others are damaged. One has a deep staining nucleus. The granules are irregularly arranged and the whole cytoplasm of one cell stains darkly. The rat died at once after a 1000 volt continuous circuit application for 1 second.

FIG. 19. Cells of the superior olive. The rat survived an instantaneous, 1000 volt continuous circuit contact for 4 days. No cell in this group is normal.

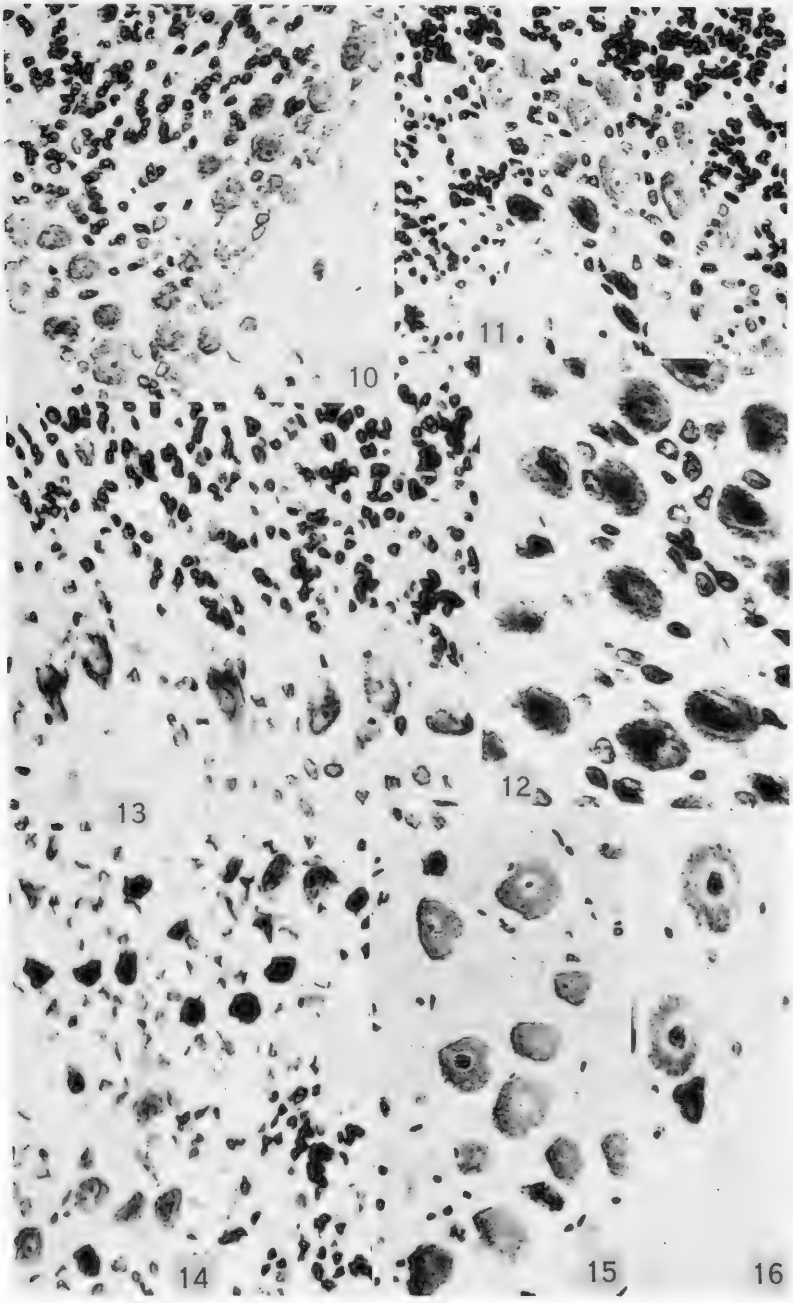
FIG. 20. Dorsal nucleus of the vagus. This rat lived 6 days after an instantaneous, 1000 volt continuous circuit injury. The cell in the lower portion of the field is normal. At the upper edge a pyknotic cell is seen. Between these are shadows of two disintegrating cells.

FIG. 21. The distinction between normal and pyknotic cells in the dorsal nucleus of the vagus is clear. The rat lived 4 days after instantaneous contact with a 1000 volt continuous circuit.

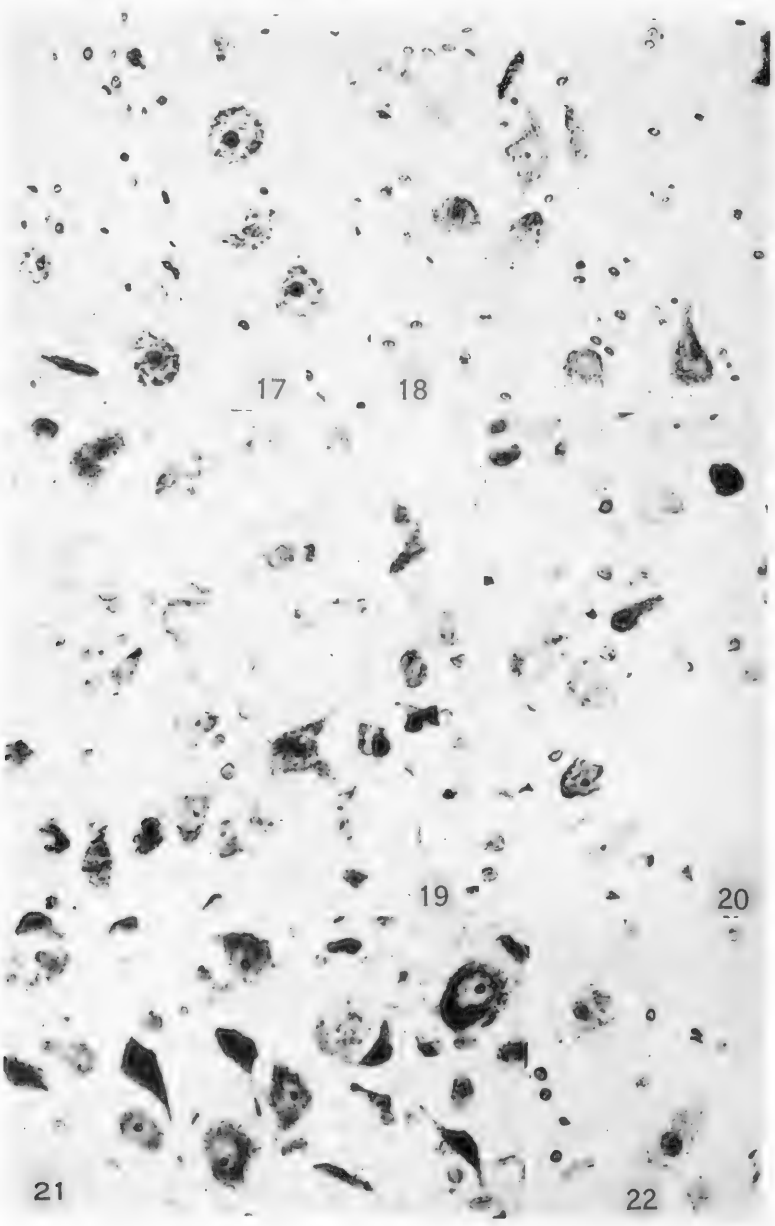
FIG. 22. These cells of the dorsal nucleus of the vagus show severe injury of both the nucleus and the cytoplasm. The rat died at once after contact with a 1000 volt continuous circuit for 1 second.



(Langworthy: Electrical injuries to nervous system)



(Langworthy: Electrical injuries to nervous system)



(Langworthy: Electrical injuries to nervous system)

INFECTIOUS MYXOMATOSIS OF RABBITS

Observations on the Pathological Changes Induced by Virus myxomatosum (Sanarelli)

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Plates 34 to 37

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In 1927, a preliminary report (1) was made concerning certain features of a disease designated by Sanarelli as infectious myxomatosis of rabbits. The purpose of the present communication is to amplify the previous report and to record some results of subsequent work.

Infectious myxomatosis is a malady indigenous to rabbits of South America and was first described by Sanarelli (2) in 1898. The disease, as he saw it, was characterized by the presence of nodules in the skin in the neighborhood of the eyes, nose, mouth, ears, and genitalia. A conjunctivitis, accompanied by a profuse purulent discharge, resulted from the involvement of the skin around the eyes. The disease ran a rapid course, death of the animals usually occurring within 1 or 2 weeks after infection. Upon palpation the tumors were firm and on section revealed a gelatinous consistency. Histologically they were found to be comprised of gelatinous material, large stellate cells, and blood vessels. The lymph nodes and spleen were enlarged, and, in histological preparations of the latter, nests of the large stellate cells were observed. The presence of the virus was demonstrated in the secretions from the eyes, in the tumors, in the various organs, and in the blood. Besides spreading spontaneously, the malady proved to be transmissible by experimental inoculation. Many kinds of animals, including man, were inoculated. The rabbit, however, was the only susceptible host found. Sanarelli was unable to cultivate the etiological agent on artificial media, nor was he able to see it by means of the microscope. In view of these facts, he concluded that *Virus myxomatosum* is similar in nature to rabic virus.

Since Sanarelli's original communication only 10 papers (see bibliography) dealing with the infectious myxoma of rabbits have appeared. A detailed review of these reports, with the exception of Findlay's (11),

was made by Hobbs (10) in 1928. Therefore, only the results of previous work that are particularly interesting or that have a bearing on the experiments to be presented at this time will be described.

Moses, Hobbs, and Findlay have shown that the myxoma virus is capable of passing through filters impervious to ordinary bacteria. No worker has been able to cultivate the etiological agent on artificial media in the absence of living host cells. The rabbit is the only known susceptible host. Even the wild hares (*Lepus braziliensis*) of Brazil are resistant to the virus (Moses, 1911).

Splendore, 1909, reported the presence of trachoma-like bodies in the tumor cells. In 1911, Moses stated that he was unable to confirm Splendore's observations concerning these bodies. Aragão, 1911, recorded his observations of certain bodies, *Chlamydozoa myxomae*, within the hypertrophic nuclei of the tumor cells. In 1927, this investigator repudiated his work concerning the nuclear inclusions and stated that the etiological agent is represented by small round granules, *Strongyloplasma myxomae*, situated within the cytoplasm of infected cells. Rivers, 1927, reported that he was able to find large granular acidophilic inclusion bodies in the cytoplasm of epithelial cells covering the myxomatous masses. Hobbs, 1928, confirmed Rivers' observations concerning the presence of these inclusions in the epithelial cells. Lipschütz, 1927, described within the cytoplasm of the swollen tumor cells, the presence of many small bodies which, inasmuch as they seemed to differ from chlamydozoa and strongyloplasms, he designated *Sanarellia cuniculi*. Findlay, 1929, failed to confirm Rivers' observations regarding the presence of cytoplasmic inclusions in epithelial cells. He was able, however, to find Lipschütz's *Sanarellia cuniculi* in the tumor cells.

Although Sanarelli in 1898 described the myxomatous masses in the skin as tumors and found that in infected animals the spleen and lymph nodes were enlarged and that at times an orchitis occurred, certain workers have been unable to confirm his observations. For instance, Aragão, 1927, stated that the myxomatous swellings are not true tumors but remarkable collections of oedema due to an infectious agent. Furthermore, he observed no involvement of the lymph nodes and spleen. Findlay, 1929, reported that "the nodular lesions are thus due, not to an active proliferation of the tissue elements, but simply to the myxomatous changes in the tissues." Moreover, he found "no enlargement of the lymph-glands or spleen." Also, according to him, "the testicles, apart from congestion, were normal even when the scrotum was affected."

From the above brief review of the reports dealing with infectious myxomatosis of rabbits, it is obvious that many conflicting statements and opinions exist. Nevertheless, the disease is so characteristic and so fatal that there is no doubt but that all of the investigators studied the same malady. In fact, many of them were dealing with the same

strain of virus, which originally came from the Oswaldo Cruz Institute in Brazil.

In a preliminary report, 1927, I described for the first time certain changes observed in epidermal cells covering myxomatous masses induced by *Virus myxomatosum*:

> Upon microscopic examination the first change noted in the epidermal cells is an increase in their size. Then, small pink, granular areas appear in the cytoplasm. These areas rapidly increase in size and frequently involve most of the cytoplasm. In the center of the acidophilic masses, blue, round or rod-shaped bodies are often seen. . . . The disease process in the epidermal cells progresses until there is complete dissolution of the cells. At this time distinct vesicles appear in the epidermis. . . .

Because of the involvement of the epidermis, a fact that had not been observed previously, I raised the question in my preliminary note as to whether I was dealing with more than one virus. Since that time work on the myxoma has been continued and now it seems advisable to record certain observations that may be of interest.

Methods and Materials

Virus.—The myxoma virus was obtained by Dr. C. E. Simon from Dr. A Moses of the Oswaldo Cruz Institute, Brazil. In May, 1926, Dr. Simon sent the virus to Dr. A. Carrel who gave it to me for study.

Methods of Inoculation.—Animals were inoculated epidermally, intradermally, subcutaneously, intravenously, and intranasally. A few animals were allowed to contract the disease through contact with infected rabbits. Except for the tumors that arose at the sites of inoculation, the disease picture was the same following all types of inoculation.

Cultures.—Infectious blood and bits of the tumors and different organs were tested for sterility by means of the usual aerobic and anaerobic cultures. No bacterium of etiological significance was encountered. In approximately 50 per cent of the rabbits that died of the myxoma, *P. lepiseptica* was obtained from some organ or tissue. Sufficient animals, however, with sterile tumor masses, blood, and organs were studied to convince one that the changes to be described in this paper were not induced by *P. lepiseptica*. In working with the myxoma, one should endeavor to use rabbits that are not carriers of *P. lepiseptica*.

Fixation and Staining.—Tissues used for histological studies were obtained from rabbits sacrificed by the intravenous injection of air. The tissues were fixed in Zenker's (5 per cent acetic acid) fluid and stained with eosin and methylene blue and according to Giemsa's method.

EXPERIMENTAL

The course of infectious myxomatosis and the histological findings are not materially influenced by the manner in which the disease is contracted. When the virus is rapidly passed in series from rabbit to rabbit, however, it kills the animals so promptly that sufficient time does not elapse for the formation of large and characteristic lesions. Therefore, if large tumors and metastases are desired for study, a virus that has been stored for a long time should be employed.

During a period of 4 years, approximately 100 rabbits have been used for various studies of the myxoma virus. Of these animals, 25 have been carefully autopsied and the tissues have been examined histologically. The results of each autopsy will not be given in detail. The findings in different tissues and organs will be described and the relative frequency of the involvement of these organs and tissues will be noted.

Skin

In every susceptible animal the epidermis, corium, and subcutaneous tissues were involved. The appearance of primary and secondary lesions in the skin is illustrated in Figs. 1–7. Such lesions are elevated, round or oval masses, the centers of which may take on a purplish color. Upon palpation the masses are firm and at times have the consistency of cartilage. The affected skin around the genitalia, however, frequently presents the appearance of oedematous tissue (Figs. 6, 7). If the lesions have progressed sufficiently, evidences of vesiculation can be found (Figs. 1, 5). In fact, fluid can be obtained from the vesicles by means of capillary pipettes. If the animals survive long enough, the vesicles are replaced by crusts which cap the tumor nodules (Fig. 5).

On sectioning the tumors one finds them firm, and hard to cut. The epidermis is thickened or shows evidences of vesiculation. The corium and subcutaneous tissues consist of a tough, pinkish, gelatinous material freely supplied with blood vessels. The tumors may be attached to the underlying muscles.

Histological examination reveals the following course of events in the epidermis. The cells increase in number and size (Fig. 9). Then small acidophilic granules appear in the cytoplasm. These rapidly increase in number and eventually replace the major portion of the normal cytoplasm. Among the acidophilic granules round or rod-shaped blue bodies may be seen (Figs. 21, 23). The nuclei become swollen or vacuolated (Fig. 23) and the chromatin is fragmented. Finally the cells undergo dissolution and vesicles appear in the epidermis (Figs. 10, 11).

In the involved corium and subcutaneous tissues an amorphous-looking material is seen in which large stellate or polygonal cells (Figs. 12, 24, 25), many polymor-

phonuclear leucocytes, and multinucleated cells (Figs. 12, 14) are found. The nuclei of the polygonal cells are swollen, and the chromatin is fragmented (Fig. 25). Dividing cells and mitotic figures are not numerous, but they do occur. Many of the so-called tumor cells are strikingly phagocytic. The cytoplasm of these cells contains a large number of granules. Whether some of the granules are similar to those described by Lipschütz and Findlay is not known. It seems, however, that the majority of the bodies represents not virus particles, but ingested material. The myxomatous masses are abundantly supplied with blood vessels, many of which are surrounded by the large polygonal cells (Fig. 12). At times the endothelial cells of the capillaries also seem to be involved (Figs. 12, 13) and are not unlike the large cells seen in other parts of the tumor.

TEXT-FIG. 1. Chain of axillary lymph nodes draining site of primary inoculation of the skin with myxoma virus. The glands were hypertrophic, hemorrhagic and firm. Histological examination of the 4 larger glands revealed the fact that the lymphocytes had been completely replaced by "myxoma" cells. Natural size.

In the epidermis and in the corium affected by the virus it appears that more than a myxomatous metaplasia of the cells already present occurs. Evidences of growth and destruction of cells are found.

Lymph Glands

Examination of the axillary and popliteal lymph glands was a routine procedure of each autopsy. If the animal is inoculated in the skin over the lateral surface of the thorax and upper part of the abdomen, it is usual to find the axillary glands on the same side involved. Glands in the other axilla and in the popliteal spaces, in a great many instances, also show evidences of involvement. A marked degree of hypertrophy is the first change noted; at times the increase in size is tenfold. Then discrete red areas appear on the surface of the glands. Finally the nodes become very firm and hemorrhagic throughout (Text-fig. 1).

Histological examination reveals that the early changes in the lymph glands are characterized by an increase in the lymphatic tissue, *i.e.*, lymphocytes. Then in the lymph sinuses around the periphery of the glands phagocytic cells make their appearance (Fig. 18), many of which are multinucleated. Soon the lymphocytes begin to disappear, and, if sufficient time elapses, the glands become devoid of such cells. At this stage of the disease, the large lymph nodes consist of cells resembling those found in the subcutaneous myxomatous nodules (Fig. 19). These cells are not normal; they have hypertrophic nuclei with fragmented chromatin, and in their cytoplasm different kinds of granules are present, many of which seem to have been ingested. On studying the sections one obtains the impression that these cells arise from the reticulum of the glands. Mitotic figures are not numerous. Nevertheless, they do occur. If the cells that comprise these "metastases" or altered lymph glands arise from the reticulum, their number can be accounted for only upon the assumption that a multiplication of the cells of the reticulum takes place under the stimulus of the virus. In addition to the type of cells just described, many polymorphonuclear leucocytes are seen.

Spleen

The spleen may be enlarged or it may be of normal size. On histological examination nests of large stellate cells are not infrequently observed. In two rabbits, many of the blood vessels in the spleen were surrounded by myxomatous tissue (Figs. 17, 20) composed of cells similar to those described in the "metastases" in the lymph glands. Around each myxomatous nodule was a compact ring of cells, many of which seemed to be spleen cells crowded together by the growth of the myxoma. Other cells in this dense mass doubtless represented the results of host reaction to the infection.

Lungs

As a rule, lesions characteristic of the myxoma were not observed in the lungs. Occasionally a pneumonic process caused by *P. lepiseptica* was encountered. In sections of the lungs from one rabbit, however, numerous myxomatous nodules (Fig. 15) were seen in the vicinity of blood vessels and bronchi. The myxomatous tissue was similar in nature to that observed elsewere in the body. Moreover, the epithelial cells of the bronchi in close proximity to the myxomas showed evidences of hyperplasia, and, in the cytoplasma of these abnormal cells, changes similar to those seen in epidermal cells were noted.

Inasmuch as the myxoma virus is regularly found in the secretions from the eyes and nose and in the blood, it is difficult to state in what manner the virus that caused the lesions in the lungs obtained entrance.

Testicles

In the majority of the rabbits the scrotal skin is affected, but it is unusual for the testicle to be attacked by the virus. In 4 rabbits, however, myxomatous

changes in the interstitial tissue of the testicle accompanied by a necrosis of the tubules was encountered.

Epididymis

The epididymis (Fig. 8) is involved more frequently than is the testicle. Within the connective tissue, myxomatous masses similar to those in the subcutaneous tissue are found. In one rabbit the epithelial cells lining different parts of the epididymis showed intracellular changes similar to those described in the epidermal cells, *i.e.*, an increase in number and size, and the presence of acidophilic cytoplasmic inclusions (Figs. 16, 22).

Tunica Vaginalis

It is not unusual to find hemorrhagic myxomatous nodules in the tunica vaginalis (Fig. 8).

Ovary and Uterus

Autopsies were performed on 3 female rabbits inoculated with myxoma virus. No lesions were found in the ovaries and uterus. If a large number of infected female rabbits were examined, it is possible that lesions might be encountered in these organs.

Pancreas, Liver, Kidney, Adrenals

No myxomatous nodules were observed in the pancreas, kidney, liver and adrenals.

Concerning the Presence of More than One Virus

The peculiar changes in the epidermis that accompany the myxomatous masses in the underlying tissue induced me to raise the question in my preliminary note (1) whether the observed pathological picture resulted from the combined activity of two viruses. Findlay (11), failing to find the changes in the epidermis described by me, again raised the question as to whether I was dealing with two viruses, one of which is indigenous to American rabbits and is not found in British rabbits. Furthermore, he suggested that one of the agents may be so labile that it is unable to withstand shipment from America to England.

During the last 4 years, I have tried in many ways to demonstrate the presence of more than one virus in the myxoma material and have been unable to do so. Vaccine virus and Virus III have been elimi-

nated as possible contaminants by passage of the myxoma through rabbits immune to these agents. Fowl-pox also is not a contaminant, inasmuch as an emulsion of the myxomatous tissue will produce no disease in fowls. The myxomatous material is innocuous for mice, rats, guinea pigs, and monkeys. Therefore, if two viruses are present in the myxomas, both are specific for the rabbit.

In view of the fact that different viruses are not equally stable, an emulsion of myxomatous tissue in 50 per cent glycerol, and citrated infectious blood were stored on ice for 1 and 2 years respectively in the hope of eliminating in this manner one of the viruses in case two were present. Lesions resulting from inoculations with these materials showed the phenomena in the epidermis that gave rise to the question regarding the presence of two viruses. Therefore, if there are two viruses in the material with which I have been working, then both are extremely resistant to aging and storage or a large number of the rabbits that I have inoculated during the past 4 years have been carriers of the second or contaminating virus. The facts that I have adduced do not conclusively exclude the possibility of the presence of two viruses. Nevertheless, in view of them, such a possibility seems unlikely.

DISCUSSION

The fact that maladies induced by viruses are characterized by hyperplasia and necrosis was discussed in a previous paper (12). In the majority of the virus diseases both of these phenomena are observed. In some, however, hyperplasia may play the important rôle, while in others necrosis dominates the picture. Such a lack of balance between the growth and destruction of tissue accounts for the fact that the activity of some viruses gives rise to certain kinds of tumors, *e.g.*, Rous' sarcoma, while the operation of others lead to vesicular lesions, *e.g.*, variola and foot-and-mouth disease.

Many of the viruses are highly species specific. Virus III attacks only rabbits; the salivary-gland virus is active only in the guinea pig; hog cholera is infectious only for swine; each polyhedral disease has its specific caterpillar host; the tumor-forming activity of the Rous sarcoma seems to be limited to chickens; certain mosaic viruses produce pathological changes in one kind of plant. This marked degree

of specificity, however, is not exhibited by all viruses. For instance, vaccinia and rabies may be induced in many kinds of animals.

A close relation exists between the viruses and host cells. This fact is emphasized by the lack of evidence to demonstrate that these active agents are capable of multiplication in the absence of living susceptible host cells, and by the presence in many instances of specific inclusion bodies in the affected cells. The relation between the viruses and cells may be not only close but specific in that certain types of cells alone are directly injured by the disease-inciting agent. For example, in rabies and poliomyelitis, it appears that nerve cells are the susceptible elements. Such cell specificity however, is not observed in vaccinia and herpes febrilis, diseases in which cells of ectodermal and mesodermal origin are involved.

The ease with which virus diseases spread from one host to another varies. Some are contagious, *e.g.*, variola and foot-and-mouth disease; others are only inoculable, *e.g.*, rabies and Rous' sarcoma; while yet others are capable of being transferred only by means of grafts, as is the case with certain infectious chloroses of plants.

The infectious myxoma of rabbits described in the present paper is one of the first maladies placed in the virus group. Moreover, when the available facts are closely examined, it is found to be one of the most interesting and characteristic of the lot. It is highly species specific, contagious, attacks cells of ectodermal and mesodermal origin, and causes hyperplasia and necrosis—the predominance of necrosis in the epidermis leads to vesicles, while the preponderance of hyperplasia in the subcutaneous and other tissues results in tumor-like masses. Moreover, inclusion bodies are found in epithelial cells involved. Finally, the causal agent is filterable, has not been cultivated in the absence of living host cells, and is very resistant to the action of certain chemicals and to long periods of storage.

Infectious myxomatosis, acquired spontaneously or induced experimentally, regularly exhibits foci of metastatic activity of the causal agent. These are always seen in the skin, particularly around the eyes, mouth, nose, and genitalia. The lymph glands draining the site of inoculation are usually involved. Other lymph nodes also may be affected. The epididymis and testicle with its tunic are not infrequently attacked. In an occasional animal, many metastases occur

around the blood vessels of the spleen and rarely multiple metastases are found in the lungs. Inasmuch as the virus is always found in the blood, the word metastases, when used in connection with this disease, does not necessarily mean that multiple lesions arise through the transportation of affected cells from one part of the body to another. The metastases described above probably represent the results of the activity of the virus operating in different parts of the animal.

There is no adequate explanation of the fact that Hobbs and I found changes in the epidermal cells associated with cytoplasmic inclusions, while Findlay in England observed none. Nor is it easy to understand why only a few workers have noted involvement of the lymph glands. In any event, the results of the work reported at the present time indicate that the rabbits in New York over a period of 4 years when infected with *Virus myxomatosum* as a rule exhibited involvement of the lymph glands.

The exact nature of the stellate, polygonal, or myxomatous cells is not known. Lipschütz, (9) speaks of them as histiocytes, while Findlay (11) describes them as "hypertrophied connective-tissue cells." Furthermore, the nature of the inclusions in the epithelial (1) and connective tissue cells (9, 11) is still an open question, as is the case with many of the inclusion bodies in the virus diseases.

I can not agree with the statement of Findlay (11) and Aragão (8) that no active proliferation of tissue elements results from infection with the myxoma virus. The evidence obtained from my work is convincing that both growth and destruction of tissues occur in this disease. Of course the cells evidencing multiplication may be those already present in the tissues. Moreover, a myxomatous change may take place in these cells as a result of the virus activity, but such an occurrence does not alter the fact that an increase in the number of cells also results from the operation of the virus and that this increase plays a part in the formation of the nodules or tumor-like masses. These facts, however, are not proof that the myxomatous masses are true neoplasms similar to cancer in man. In fact, some investigators are not convinced that the exact relation of Rous' sarcoma to true neoplasms has been determined.

In certain respects the myxoma of rabbits resembles the Rous sarcoma of chickens. In others, however, it is quite different. If, as

some believe, the Rous sarcoma appears to be more closely related to true neoplasms than to diseases induced by highly contagious agents, then the myxoma, upon further study, may serve to bridge the gap between the Rous tumor and other virus maladies, and to indicate that, after all, no great difference exists between tumor-forming viruses and those causing vesicular or destructive lesions.

SUMMARY

The virus of infectious myxomatosis of rabbits (Sanarelli) induces multiple lesions in the skin, lymph glands, tunica vaginalis, epididymis, testicle, spleen, and lungs.

Growth and destruction of cells in the epidermis overlying the myxomatous masses leads to the formation of vesicles. Cytoplasmic inclusions are found in affected epidermal cells. Occasionally, similar inclusions are seen in other involved epithelial cells. The nature of the inclusions is an open question.

In the myxomatous masses situated in the subcutaneous and other tissues, evidences of alteration and growth of certain cells are observed.

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EXPLANATION OF PLATES

PLATE 34

FIG. 1. Primary "tumor" surrounded by secondary nodules. Several vesicles cap the primary growth.

FIG. 2. Multiple primary tumors induced by rubbing the virus on the freshly shaved skin. The central portions of some of the nodules reveal a purplish tint.

FIG. 3. Involvement of the eyelids accompanied by a white, purulent discharge.

FIG. 4. Metastatic nodule in lip.

FIG. 5. Metastatic nodule in skin over shoulder. The growth is capped by a vesicle undergoing desiccation.

FIG. 6. Oedema around the vaginal orifice.

FIG. 7. Oedema of prepuce and scrotum.

FIG. 8. Four metastatic nodules, three in the tunic, one in the epididymis.

PLATE 35

FIGS. 9, 10, 11. Involvement of epidermis characterized by hyperplasia of the epithelial cells followed by necrosis resulting in vesicles. × 375, × 125, × 125 respectively.

FIG. 12. Section through a subcutaneous nodule comprised of gelatinous material, large polygonal cells, giant cells, blood vessels, and polymorphonuclear leucocytes. × 250.

FIG. 13. Enlargement of capillary, indicated by arrow in Fig. 12, showing involvement of endothelial cells. × 1000.

FIG. 14. Higher magnification of giant cell shown in Fig. 12. × 1000.

PLATE 36

FIG. 15. Metastasis in the lung. The epithelium of the bronchus near the myxomatous tissue is hyperplastic and contains cytoplasmic inclusions. × 150.

FIG. 16. Represents the involvement of epithelial cells in the epididymis which is characterized by hyperplasia and cytoplasmic inclusions. Compare with Fig. 22. × 300.

FIG. 17. Metastasis around blood vessel in spleen. × 125.

FIG. 18. Early lesion in lymph node. Note changes in cells lining the lymph sinus and the presence of large phagocytic cells within the sinus. × 250.

FIG. 19. Complete replacement of lymph node by myxomatous tissue. × 250.

PLATE 37

FIG. 20. Metastasis in spleen. Eosin and methylene blue. × 125.

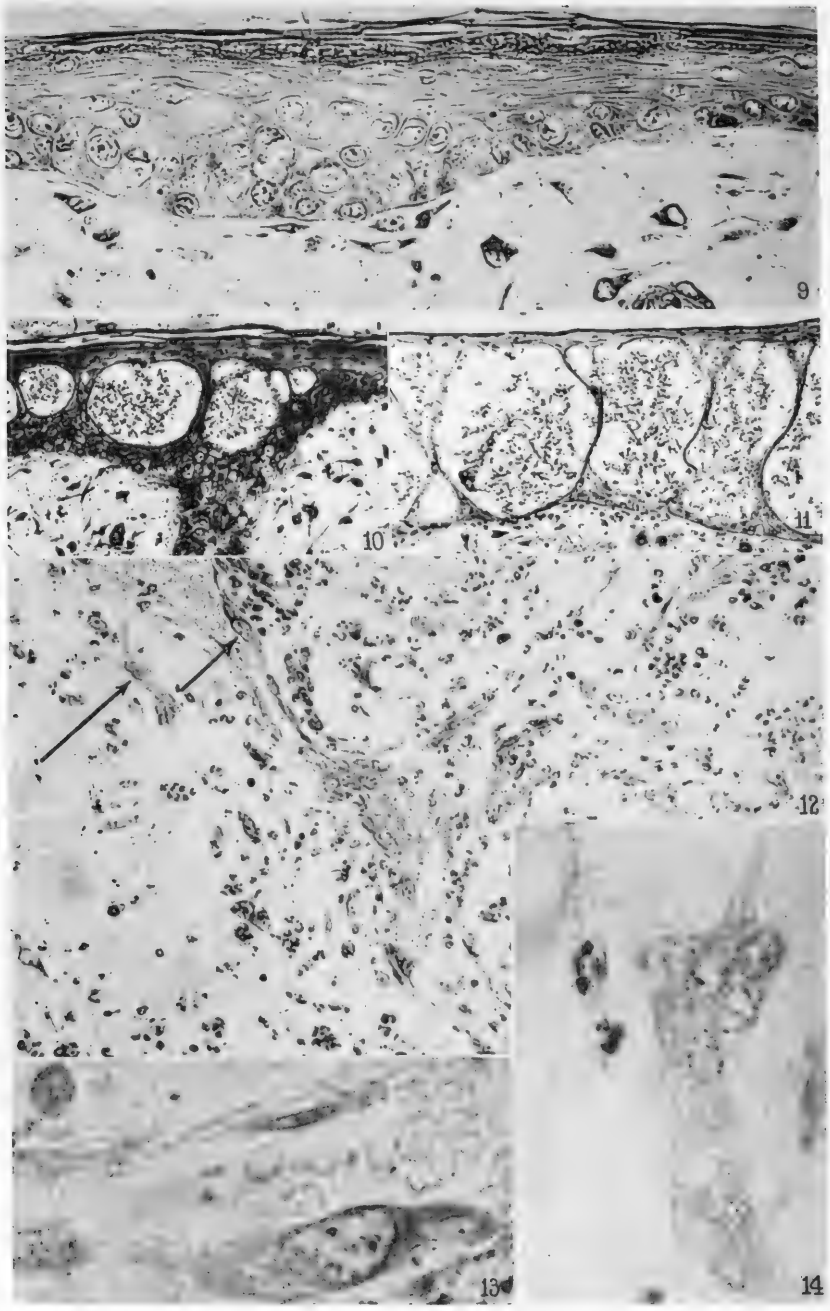
FIG. 21. Acidophilic cytoplasmic inclusions in epidermal cells. Eosin and methylene blue. × 800.

FIG. 22. Acidophilic cytoplasmic inclusions in epithelial cells of epididymis. Compare with Fig. 16. Eosin and methylene blue. × 1000.

FIG. 23. Epidermal cell with vacuolated nucleus and granular acidophilic cytoplasmic inclusion in which are situated three blue coccoid bodies. Giemsa. × 1500.

FIGS. 24, 25. Myxoma cells. Note fragmentation of chromatin. Giemsa, and eosin and methylene blue respectively. × 1500.

(Rivers: Infectious myxomatosis of rabbits)



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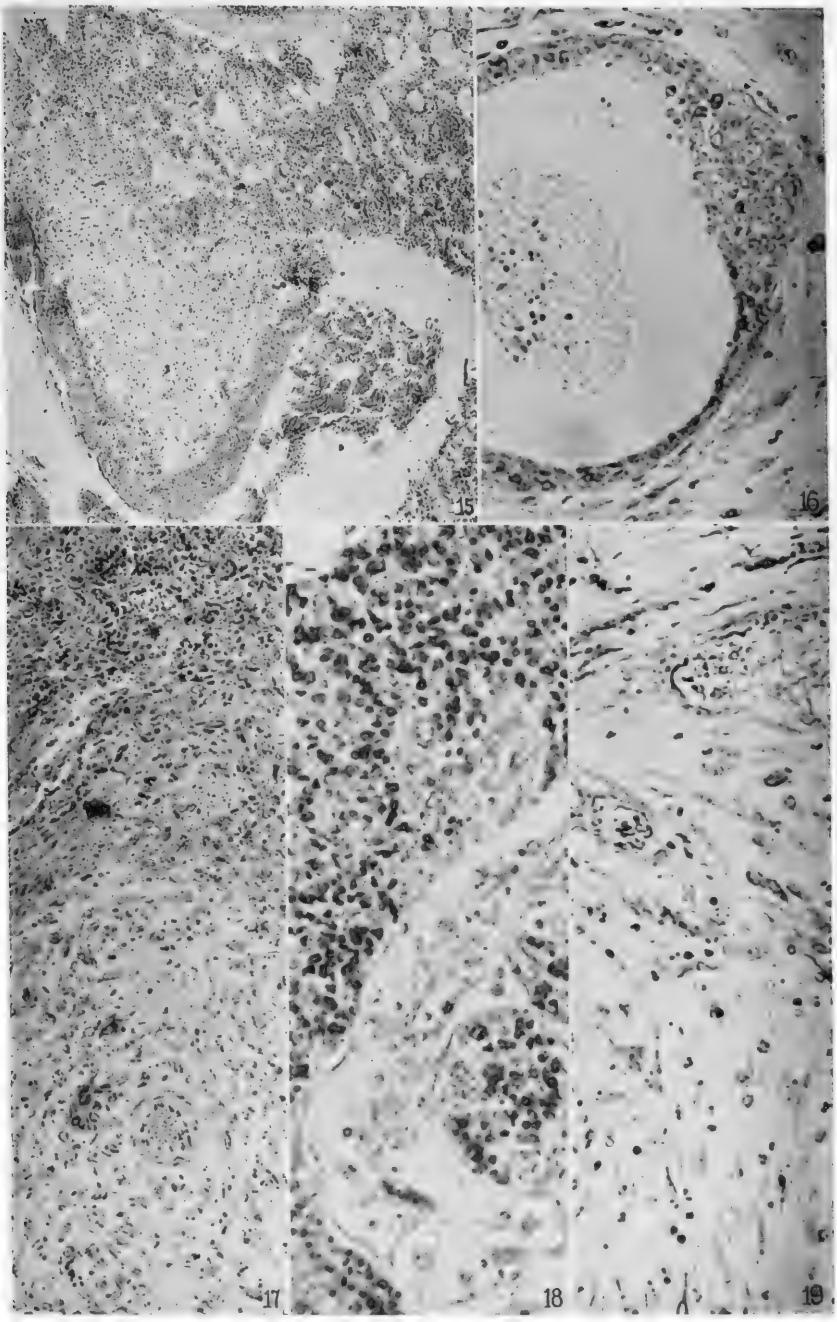

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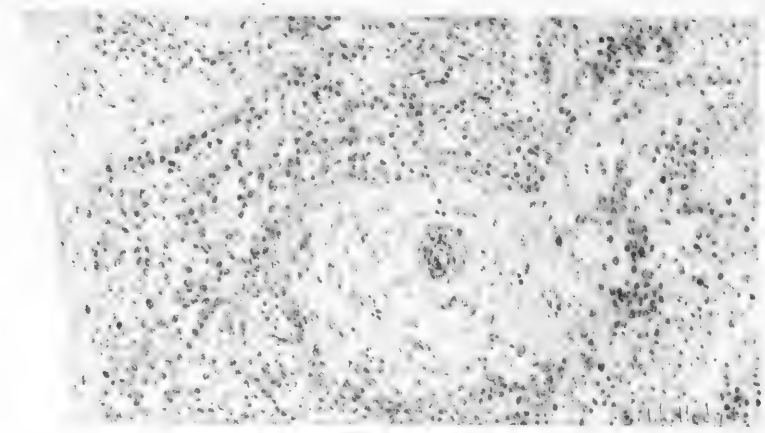

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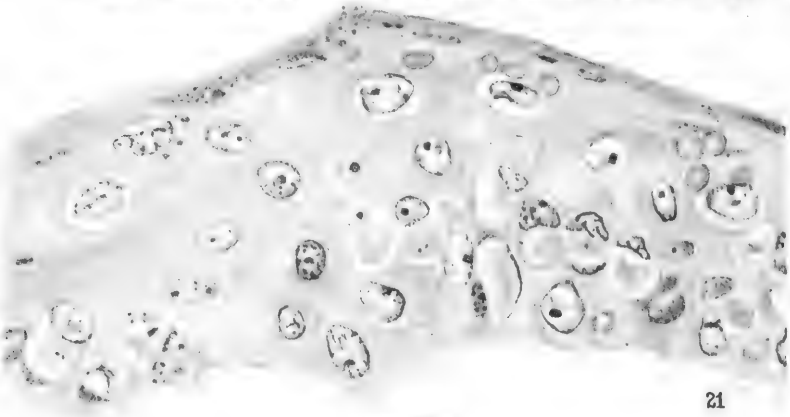

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